FACTORS AFFECTING POST-THAW QUALITY OF CRYOPRESERVED BOAR SPERM AND ITS EFFECT ON GILT FERTILITY

BY

KELLI ANN MCNAMARA

THESIS

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Master's Committee:

Associate Professor Robert V. Knox, Chair
Professor Matthew B. Wheeler
Professor David J. Miller

ABSTRACT

In several species, cryopreservation of sperm has played a major role in genetic advancement as well as conservation of genetic resources. However, due to variability in post-thaw quality of sperm and decreased pregnancy rates and litter sizes, the swine industry has not been able to fully utilize the potential of frozen-thawed boar sperm (FTS). Two experiments were performed to evaluate factors affecting the fertility of FTS.

Experiment one determined the effect of thawing rate of FTS and its effect on post-thaw quality. Ejaculates (n = 15) used were obtained from 13 boars and frozen from March 2010 to March 2011. Samples were frozen in 0.5 mL straws at a concentration of 1.4 x 10^9 sperm/mL. To test for the effects of thawing temperature and duration of thawing, sub-samples from individual straws from each ejaculate were assigned to each treatment. The treatments included straws (n = 15/treatment) thawed at 50 °C for 10, 20, and 30 s or thawed at 70°C for 5, 10, and 20 s. Following thawing, samples were evaluated for post-thaw motility (PTM), viability (VIA), and intact acrosomes (IA) at 5, 30, and 60 min. Data were analyzed using the MIXED procedures of SAS for the effects of temperature, duration of thawing, and boar. There was an effect of treatment ($P < 0.0001$) on PTM, VIA, and IA, and an effect of storage time ($P < 0.0001$) on PTM and IA. Treatment influenced PTM with thawing at 70 °C for 20 s having reduced PTM (3.4 ± 0.5%) compared to the other treatments (41.3 ± 0.7%). There was also an effect of treatment ($P < 0.0001$) on VIA with 70 °C for 20 s resulting in the lowest (5.8 ± 0.8%) and 70 °C for 5 s also showing lower VIA (50.2 ± 1.6%) compared to all other treatments (55.8 ± 1.3%). Treatment also influenced IA ($P < 0.0001$) with 70 °C for 20 s showing reduced IA (61.9 ± 1.8%) compared to the other treatments (79.9 ± 1.1%). These results indicate that when thawing

boar sperm in 0.5 mL straws with the cryoprotectant used, 70 °C for 20 s and 5 s reduced sperm fertility, while thawing at 70 °C for 10 s and 50 °C for 10 to 30 s resulted in no effect on PTM, VIA, and IA. However, it would appear that 50 °C for 20 s provided the greatest safety margin with the highest fertility measures.

Experiment two was performed to determine the effect of variation in the post-thaw motility of FTS on pregnancy rate, litter size, and fetal paternity when used in the 1st or 2nd insemination. Ejaculates from 38 boars were collected and frozen in 0.5 mL straws. Upon thawing at 50° C for 20 s, samples were classified (mean ± SEM) by motility as Poor (P, 20.2 ± 1.1%), Moderate (M, 31.3 ± 0.9%), or Good (G, 43.5 ± 0.8%). In 7 replicates, mature gilts were synchronized and checked for estrus at 12 h intervals and assigned at estrus (n = 207) to receive 4.0 billion total sperm in each AI at 24 and 36 h after onset of estrus using the treatments: 1) P and M (P-M); 2) M and P (M-P); 3) G and M (G-M); and 4) M and G (M-G). For each treatment combination, a set of three boars were randomly selected within motility class for their allelic distinction with M sperm from a single boar represented across all treatments and sires used in both 1st and 2nd inseminations. Inseminations occurred at 24 and 36 h following estrus, and insemination to ovulation interval (IOI) was determined using ultrasound at 12 h intervals. Reproductive tracts were collected at 32 ± 1.0 d following AI. Treatment did not interact with IOI ($P > 0.10$) and did not affect ($P > 0.10$) pregnancy rate (57, 67, 71, 76 ± 7.2%, pooled SEM) or total number of fetuses (9.2, 9.1, 9.5, 10.0 ± 0.8) for P-M, M-P, G-M, and M-G treatments, respectively. Treatment did affect ($P < 0.05$) the number of fetuses sired from the 1st AI (3.1, 7.2, 6.4, 6.3 ± 1.2) and 2nd AI (5.7, 2.6, 3.0, 3.6 ± 0.9) for the P-M, M-P, G-M, and M-G treatments, respectively. The IOI also affected ($P < 0.05$) the proportion of offspring sired by the 2nd AI (30.0, 57.7, 51.3, 18.3, ± 6.5%), as well as the number of fetuses sired by each AI.

The results of these studies show that thawing frozen boar sperm in 0.5 mL straws can be accomplished at 70 °C for 10 s or 50 °C for 10, 20, or 30 s, however we recommend thawing at 50 °C for 20 s to provide a window for less than optimal temperature and time of thawing. In addition, our data suggest that use of poor motility sperm in the first or second AI will sire fewer offspring than higher quality sperm, but moderate and good motility sperm optimized the number of pigs sired and offspring produced. It was also shown that the first AI sired a greater number of pigs in most cases when FTS was used in a double insemination in mature gilts.

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1. Worldwide Commercial Swine Production

In the global market, pork accounts for greater than 40% of red meat consumed annually (Gerrits et al., 2005). China is the leader in both pork production and consumption. Other leaders in pork production include the USA, the EU, Brazil, Canada, and Mexico, while Japan is the leading importer of pork products (USDA, 2013). Gerrits et al. (2005) estimated that by the year 2020, global pork demand will reach more than 120 million tons, with most of the consumption occurring in developing countries. It has also been projected that among leading importers, consumption of pork products will increase from 5,411 tons to greater than 6,200 tons by the year 2022 (USDA, 2013). With this projected increase in global pork consumption, swine producers must be able to increase production and production efficiency in order to accommodate the projected growth in global demand.

To meet the demand for world pork consumption, the swine industry has accepted and come to rely on various technologies that increase and improve production efficiency at the commercial level. In particular, artificial insemination (AI) is the best example of a technology that has been rapidly adopted in the swine industry and has arguably been one of the most important management tools leading to improved herd productivity (Bailey et al., 2008). In 1990, approximately 7% of sows in the United States were bred using AI, and this number has increased to approximately 90% (Didion, 2008). AI is a common breeding tool used on most large European and North American pig farms (Bolarín et al., 2006), although the extent of its use in different countries is highly variable (Gadea, 2003).

Over the past 20 years, swine production and management systems in the United States have undergone several significant changes. Foremost among these changes has been the transition from large numbers of small, family owned farms with less than 2,000 head, to fewer numbers of large commercial operations with more than 2,000 head. While total pig production has not changed, the number of swine operations decreased from 240,000 (1992) to 70,000 (2004) as the shift to larger farms continued (Key and McBride, 2007). Additional changes that have had a large impact on swine production has involved changes in confinement swine housing, biosecurity changes for improved herd health management, and the advances that have come in reproductive management with the adoption of AI. Collectively, these and other changes have helped increase the efficiency of pork production for producers (Gerrits et al., 2005; Key and McBride, 2007).

One of the most important factors driving the progress in swine production toward more profitable swine operations is reproductive efficiency. Artificial insemination allows for greater efficiencies in gene transfer, labor, and costs when compared to natural service. While developed in the early 1970s, the current use of AI with liquid extended semen accounts for greater than 90% of all inseminations performed (Johnson et al., 2000; Weitze, 2000). In the international market, artificial insemination has shown rapid growth throughout Europe, East Asia, and South America (Riesenbeck, 2011) due to the realization of the advantages of utilizing this technology. These primary advantages recognized include greater reproductive efficiency through the ability to disseminate a single ejaculate from a superior sire to multiple females great distances away, while helping to limit the spread and risk of disease, and to take advantage of labor and cost efficiency (Riesenbeck, 2011).

2. Artificial Insemination in Swine

This review of literature is focused toward the use and fertility of frozen thawed boar sperm. However, before a discussion of that topic, it is relevant and important to summarize the current state of swine insemination technology and the physiology surrounding these events.

2.1 Industry Transition to Artificial Insemination

Worldwide commercial application of AI use in swine production did not take place until the 1980s when insemination protocols were standardized (Gadea, 2003). From 1990 to 2000 there was an increase in the development of field AI services in many countries. In some countries, the percentage of sows inseminated artificially is greater than 80% (Weitze, 2000). More than 99% of these inseminations are made with semen extended in a liquid state and usually stored at 15-20 °C for up to 3 days (Roca et al., 2006b). In practice, 3 billion motile spermatozoa in a volume of 80-100 mL of extender are inseminated into the sow two to three times during standing estrus (Tummaruk et al., 2007). This procedure of insemination with fresh semen has become prevalent in most countries (Riesenbeck, 2011), with approximately 25 million AIs registered worldwide every year (Roca et al., 2006b).

Artificial insemination in sows has provided multiple benefits compared to natural service. Firstly, it reduces labor necessary to collect boars in comparison to the labor necessary to manage boars with natural service. Secondly, it greatly reduces the time needed for mating and allows for the insemination of many females in a short period of time (Flowers and Alhusen, 1992). The utilization of AI has allowed producers to greatly improve herd genetics through the

dissemination of genes from superior sires to an increased number of females (Bailey et al., 2008; Colenbrander et al., 2000; Maes et al., 2008; Roca et al., 2006b). AI has been shown to reduce the risk of disease transmission, as any disease that may be carried and shed by the boar, whether through the testes, other tissues of the genital tract, preputial fluids, or respiratory secretions, does not come into contact with the female (Bailey et al., 2000; Maes et al., 2008). AI also helps reduces stress on the sow or gilt as she is not required to support the weight of the boar during mating. This is especially important in weaned sows with structural weaknesses and gilts as the weight difference between a gilt and a mature boar is usually large (Flowers and Alhusen, 1992).

A disadvantage of swine AI, compared to AI in other species, is the number of sperm needed to achieve optimum fertility. Currently, the sperm dose used is approximately 2.5-3.0 x 10^9 spermatozoa in 80-100 mL of extender (Matthijs et al., 2003); however, the industry is trying to achieve a reduction in the number of spermatozoa inseminated while still maintaining optimum fertility. Lessard et al. (2003) showed that gilts inseminated with 1.0 x 10^9 spermatozoa had similar reproductive performances to those inseminated with a commercial dose of 3.0 x 10^9 spermatozoa. When using liquid semen, the length of storage at 16 °C limits the fertile lifespan of the spermatozoa, which could shorten the optimal period for insemination relative to ovulation (Kemp et al., 1998). This shortened fertile lifespan leads to the need for more sperm in the dose in order to ensure that enough sperm reach the utero-tubal junction (UTJ) to obtain high fertility (Watson and Behan, 2002). Only about 25% of the thawed sperm present in the AI dose have the ability to progress toward the site of fertilization (Cremades et al., 2005). This could explain the improvement when a two or three-fold increase in sperm numbers per AI dose are used and how

boar fertility potential is masked when large numbers of motile sperm are used (Cremades et al., 2005; Foxcroft et al., 2008).

2.2 Physiological Responses to Artificial Insemination

2.2.1 Immune Reaction

At the time of estrus in non-mated sows, the endometrium has a large number of neutrophils, and it has the ability to respond quickly to invading agents, such as spermatozoa, after insemination. After AI, a normal uterine response is the influx of polymorphonuclear (PMN) cells into the uterine lumen (Kaeoket et al., 2001). The introduction of the inseminate appears to be the trigger for the influx of PMNs, in which phagocytosis begins almost immediately following insemination (Colenbrander et al., 2000). Phagocytosis seems to be responsible for the removal of the largest number of spermatozoa (Steverink et al., 1998). This process, along with the semen backflow after AI, results in a rapid and dramatic reduction in the number of spermatozoa in the female reproductive tract within a few hours after insemination (Colenbrander et al., 2000; Matthijs et al., 2003). The loss of spermatozoa in backflow has been shown to be reduced when the inseminate volume is reduced, which leads to a greater number of spermatozoa retained in the uterus (Matthijs et al., 2003). Another finding from Matthijs et al. (2003) showed that adding caffeine and calcium chloride ($CaCl_2$) to the insemination dose resulted in a greater number of non-phagocytosed spermatozoa in the reproductive tract of the female 4 h after insemination. This could be due to faster transport of the sperm through the

reproductive tract or due to a reduction in the recruitment of PMNs, which would contribute to the reduction in phagocytosis.

The inflammatory response to spermatozoa appears to be a normal physiological reaction that clears the uterus of pathogens and excess spermatozoa in order to prepare an optimal uterine environment for the anticipated embryos (Matthijs et al., 2000; Rozeboom et al., 1998). A study by Matthijs et al. (2000) showed the number of spermatozoa available for fertilization was greatly reduced within 4 h, with only a small percent of the inseminated number available at the time of fertilization. Insemination resulted in a rapid recruitment of PMNs which was triggered by the addition of liquid into the uterus and not triggered by the presence of sperm cells and/or seminal plasma, as sows inseminated with only extender showed the same influx of PMNs as those inseminated with both extender and sperm. In contrast, Rozeboom et al. (1998) reported that the number of PMNs recovered from gilts inseminated with semen was higher than females inseminated with extender alone. The numbers of PMNs increased continuously in the semen-treated gilts and peaked at 12 h after insemination, with inflammation persisting in the uterus for more than 24 h after AI. While PMNs are recruited in the uterus, neutrophils were not observed in the oviductal epithelium or any other segments of the oviduct (Jiwakanon et al., 2006). This indicates that once sperm reach the isthmus, ampulla, and infundibulum, they become "immunoprivileged," and the decreased recruitment of phagocytes leads to a conclusion that specific immune regulation exists in the oviduct. Matthijs et al. (2000) found that species-specific components of sow serum bind to boar sperm and can reduce phagocytosis of the sperm by PMNs. It is thought that these components have properties similar to antibodies. It was also found that sperm induced to capacitate were less likely to be phagocytosed, which indicates that

during capacitation, one of the potential changes that may occur is removal or reduction in the number of binding sites for the PMNs.

2.2.2 Sperm Reservoir Establishment

The establishment of a functional sperm reservoir in the female reproductive tract is important for fertilization of the oocytes, and the mammalian oviduct has often been described as a "holding pen" for spermatozoa as they make their way to the eggs (Holt, 2011). In the pig, the utero-tubal junction (UTJ) and the caudal isthmus act as the sperm reservoir to restrict access to the fertilization site (Hunter, 1981) and prevent excessive numbers of sperm from reaching the oocytes at a given time (Tummaruk and Tienthai, 2011) to prevent polyspermic fertilization (Petrunkina et al., 2007; Topfer-Petersen et al., 2002).

The sperm population in the reservoir depends on initial sperm quality, the site of sperm deposition, and the number of sperm inseminated (Foxcroft et al., 2008; Kemp et al., 1998; Tummaruk et al., 2007). Once inseminated, the sperm travel through the uterus and reach the reservoir, usually within minutes to one hour (Tummaruk et al., 2007), by myometrial contractions that are elicited by the distension of the uterine lumen (Saravia et al., 2005). Upon reaching the oviduct the sperm are trapped in the caudal isthmus by binding to epithelial cells lining the duct (Topfer-Petersen et al., 2002), and sperm storage in the oviduct involves adhesion to and controlled release from the epithelium (Petrunkina et al., 2007). Of particular importance is the concept that binding to the oviductal epithelium stabilizes the spermatozoa and greatly enhances their survival in a relatively hostile environment (Petrunkina et al., 2007) while also modulating sperm capacitation (Topfer-Petersen et al., 2002).

Since the timing of ovulation is not always synchronized with insemination, the sperm reservoir represents a natural mechanism that ensures the sperm and the egg meet at the optimum time. After the reservoir is filled, the release of sperm cells from the reservoir is exponential in the first few hours after insemination (Steverink et al., 1997). Capacitation of spermatozoa must be synchronized with ovulation, but since tight synchrony cannot always be achieved, a continuous supply of fertile spermatozoa must be provided so that enough are present at the actual time of ovulation (Petrunkina et al., 2007; Tummaruk et al., 2007).

Besides facilitating the constant release of fertile spermatozoa to the site of fertilization, the sperm reservoir also functions to protect sperm. The storage of sperm under conditions that maintain sperm viability to save energy is an important contribution to maximize the fertilization success, and previous research showed that an increase in dead or defective sperm cells was considerably lower in those sperm cells that were bound to the oviductal epithelium (Topfer-Petersen et al., 2002). This study showed the ability of the oviduct to select for viable and uncapacitated sperm and its ability to protect them for a period of time from being damaged or initiating capacitational changes too early. In an experiment by Waberski et al. (2006) it was found that sperm with cytoplasmic droplets have reduced binding capacity to the oviductal epithelium *in vitro*. This experiment also reported that the reduced binding was likely due to changes in the boar sperm membranes during storage, which supports the hypothesis that damaged sperm are excluded from the sperm reservoir.

2.3 Timing of Artificial Insemination on Fertility

2.3.1 Estrus Synchronization and Detection of Estrus

When using artificial insemination on commercial swine farms, it is beneficial to synchronize estrus and ovulation in order to optimize fertility, labor costs, and animal flow. This can be accomplished through weaning or the through the use of hormones. Estrus is somewhat synchronized after weaning or through the use of altrenogest treatment in cycling gilts or following estrus induction with PMSG and hCG. Ovulation induction is also used to time inseminations, and hCG, LH and GnRH have been used to aid in use of fixed-time AI procedures (Kemp et al., 1998). An approved method for synchronizing estrus in cycling females is through the use of altrenogest, a synthetic progestagen. It has been shown that gilts undergoing treatment with altrenogest show growth of large follicles 3 days following the last day of administration (Guthrie et al., 1993).

In terms of duration of altrenogest treatment, Stevenson and Davis (1982) found that treatment for either 14 or 18 days had no effect on the interval to estrus, however, the 18 d treatment produced more precise synchronization while the 14 d treatment tended to spread the distribution of estrus. In the 14 d treatment, more than 98% of the gilts were in estrus in 3 to 10 d post-treatment. They concluded that while the 18 d treatment yielded a tighter window of synchronization, the 14 d treatment was long enough for corpora lutea to regress and synchronize a fertile estrus in randomly cycling gilts. The dose of altrenogest is important to consider for synchronizing estrus. In a study by Shimatsu et al. (2004), the interval between the last dose of altrenogest and the onset of estrus was positively correlated with the dose of altrenogest

administered. In this study, a dose of 5 mg/day was not enough to control follicular growth, however, treatment with either 10 or 15 mg/day was efficient for synchronization of both estrus and ovulation. Results have shown that synchronization with altrenogest followed by three scheduled inseminations (d 5, 6, and 7 following altrenogest feeding) can lead to fertility similar to that for gilts checked twice daily for estrus and inseminated according to conventional methods (Davis et al., 1985).

Altrenogest can be used to synchronize estrus by itself or in conjunction with PG 600 (400 IU eCG + 200 IU hCG) (Horsley et al., 2005). PG 600 is used to induce estrus in prepubertal gilts and weaned sows that have not expressed estrus. Several studies have shown that PG 600 can advance onset of estrus, as well as accelerate and synchronize puberty by inducing ovulation in prepubertal gilts between 151-180 days of age (Estienne et al., 2001; Horsley et al., 2005; Iwamura et al., 1998; Martinat-Botte et al., 2011) and increase ovulation rate (Estienne et al., 2001; Horsley et al., 2005; Martinat-Botte et al., 2011).

Standard industry practice utilizes a once-daily estrus detection system using fence-line boar exposure while applying the back pressure test, with inseminations occurring at 0 and 24 h after onset of estrus (Knox et al., 2002). When the once-daily strategy is used the duration of estrus is less accurate, which can lead to inaccuracy when predicting ovulation time. Further, animals with a short duration of estrus may not be detected (Steverink et al., 1999). A study by Knox et al. (2002) showed that more frequent estrus detection coupled with delaying the first insemination by 12-16 h after onset of estrus allows more inseminations to occur within 24 h prior to ovulation, which leads to acceptable farrowing rates and litter sizes. Conversely, once daily estrus detection can optimize labor performance, as well as prevent habituation to the boar stimulus to optimize sow interest and boar excitement. To correctly perform detection of estrus,

important signs of the sow in heat can include mucous discharge, interest in the boar and other females, mounting activity, and immobility in close proximity to a boar or induction of the standing reflex when back pressure is applied (Bolarín et al., 2006). Davis et al. (1985) reported that three inseminations may be necessary in gilts to achieve acceptable fertility after estrus synchronization. Short term boar exposure prior to estrus has also been shown to improve the estrous and ovulation responses in prepubertal gilts when exposure is administered for as little as 4 d prior to PG 600 injection (Breen et al., 2005). This short term exposure can also influence the onset of puberty in gilts between 160-180 d of age. It has been shown that during courting, a mature boar produces derivatives of 16-androstene pheromones (Pearce and Hughes, 1987), which are found in the urine and saliva of the boar (Hughes et al., 1990). These pheromones were found to have a signaling function involved in stimulating puberty in gilts (Pearce and Hughes, 1987).

2.3.2 Estrus to Ovulation Interval

Significant relationships have been found between the interval from weaning to onset of estrus and the duration of estrus, as well as the duration of estrus and the estrus to ovulation interval. Sows coming into heat early after weaning typically have estrus lasting 3 days, whereas sows coming into heat on day 5 after weaning show a heat period of approximately 2 days, while those coming into heat later have very short estrus durations (Kemp et al., 1998; Knox et al., 2002; Nissen et al., 1997; Weitze et al., 1994). In order to accommodate these differences, insemination can include AI starting on day 2-3 of heat for animals expressing estrus early, while those having an average interval from weaning to estrus should be inseminated at 0 and 24 h or

24 and 36 h after estrus detection. Those animals coming into heat late should be inseminated at 0 and 24 hours (Weitze et al., 1994). Duration of estrus is a good indicator for ovulation time (Bracken et al., 2003), with a majority of sows ovulating ~70% of the way through estrus (Almeida et al., 2000; Bortolozzo et al., 2005; Garcia et al., 2007; Soede et al., 1995a; Steverink et al., 1997) with most sows ovulating between 40-48 h following estrus detection (Soede et al., 1995b; Weitze et al., 1994).

2.3.3 Insemination to Ovulation Interval

As the survival time of spermatozoa in the female reproductive tract is limited, the insemination to ovulation interval is important. When the interval between insemination and ovulation is reduced, higher the fertilization rates result (Bailey et al., 2000). When liquid semen is used, the optimal interval for insemination to ovulation in sows is between 0 and 24 h (Kemp et al., 1998; Soede et al., 1995a), with the highest fertility and maximum litter size observed when insemination occurs within 12 h prior to ovulation (Dziuk, 1970, 1996; Nissen et al., 1997). The greatest number of embryos, highest fertilization rates, and fewest unfertilized oocytes were observed when inseminations occurred within 24 h prior to ovulation regardless of low or high sperm numbers or multiple inseminations (Bracken et al., 2003). However, litter size has been reported to decrease when a single insemination was used (Bortolozzo et al., 2005). A significant reduction in the total number and number of viable embryos was seen when insemination occurred greater than 24 h prior to or 4 h after ovulation (Nissen et al., 1997), with peri-ovulatory inseminations typically resulting in the highest farrowing rates and litter sizes (Bolarín et al., 2006). However, normal fertilization can occur even when insemination takes

place more than 40 h prior to ovulation and 8 h after (Soede et al., 1995a), but often leads to reduced fertility.

Timing of AI becomes even more important when reduced numbers of spermatozoa or frozen-thawed sperm (FTS) are used, although optimal fertility seems to be a function of both sperm numbers and AI timing (Garcia et al., 2007). Using low quality FTS, Spencer et al. (2010) observed that increased pregnancy rates occurred when insemination occurred between 4 h prior to and 8 h after ovulation. The optimal time of insemination is dependent on several factors including the lifespan of a sufficient number of spermatozoa to ensure fertilization, the speed of sperm transport and sperm capacitation, and the lifespan of the oocytes (Soede et al., 1995a). Negative effects on litter size and farrowing rates are typically expected when AI is performed after ovulation (Steverink et al., 1999). It can result in polyspermic fertilization (Hunter, 1984; Soede et al., 1995b) due to hormonal changes in the female reproductive tract (Hunter, 1984) and the fact that the regulation of the number of spermatozoa released from the uterus to the site of fertilization is altered (Jiwakanon et al., 2006). This can lead to the possibility of more than one competent sperm cell penetrating the oocyte (Soede et al., 1995b; Waberski et al., 1994). Kaeoket et al. (2002) and Kaeoket et al. (2005) showed that insemination after ovulation can lead to early embryonic loss during pregnancy. This is due to a disruption in the transport of spermatozoa to the oviduct, resulting in a lower proportion of oocytes with accessory spermatozoa.

The insemination to ovulation interval is also important because oocytes, once ovulated, have a limited fertile lifespan. Once they reach the oviduct a population of capacitated spermatozoa from the sperm reservoir must be present to achieve maximum fertilization rates (Colenbrander et al., 2000; Kemp et al., 1998). It should also be noted that ovulation occurs over

a1 to 3 h period in spontaneously ovulating sows (Colenbrander et al., 2000), which means that there need to be an adequate number of spermatozoa released from the sperm reservoir to fertilize all of the oocytes as they are ovulated. Because of this, it may be best to utilize a double insemination to ensure availability of fertile spermatozoa, especially when using low numbers of less fertile boar sperm, as the life span in the female reproductive tract may be much shorter for FTS than liquid semen. The double inseminations should be administered ~10 h apart and as close to ovulation as possible (Almlid et al., 1987); however, current methodology is limited for predicting the time of ovulation with enough accuracy for the successful utilization of fixed-time insemination (Knox et al., 2011). Willenburg et al. (2003) concluded that optimal conditions for fertility in gilts may include twice-daily estrus detection, insemination times that average 3-4 min/female, AI using at least 3 billion fertile sperm, and a double insemination that provides the last insemination at 12 h prior to ovulation.

3. Advancements in Artificial Insemination Techniques

New technological advancements can assist in better prediction of ovulation time in sows through the use of real-time ultrasound (RTU), as well as aid in higher conception and farrowing rates through the use of intra-uterine (IUI) and deep intra-uterine insemination (DIUI) techniques. These technologies, as well as computer-assisted semen analysis (CASA) machines and sperm sexing systems, can lead to better management strategies for reproduction on commercial farms.

3.1Real-Time Ultrasound

Ultrasonography in pigs may be used successfully to monitor follicular growth and the estimated time of ovulation, which can help determine the interval from insemination to ovulation and how it pertains to fertilization rate, embryonic development, and farrowing rate (Nissen et al., 1997). It has been shown to be a reliable procedure that has no apparent effect on fertilization (Soede et al., 1995a). Trans-rectal ultrasound has been shown to be a valuable tool to estimate the number of pre-ovulatory follicles on each ovary. The exact number of pre-ovulatory follicles may not always be determined due to the inability to view all of them on each ovary; however, frequent scanning is helpful to determine ovulation time, as a disappearance of a number of these pre-ovulatory follicles has been used to indicate the beginning of ovulation (Bolarin et al., 2009).

3.2 Intra-Uterine and Deep Intra-Uterine Insemination

It is known that as the site of sperm deposition gets deeper into the reproductive tract, fewer sperm per insemination dose are required to maintain fertility (García et al., 2007), and uterine insemination may enable the use of low sperm doses in both gilts and sows (Bracken et al., 2003). After routine intra-cervical insemination almost 40% of the insemination dose is voided in the vagina (Colenbrander et al., 2000). With conventional AI the recommended number of sperm cells present in the insemination dose is usually in excess of 2.5 billion sperm (Roca et al., 2006b), however, the dose can be decreased when the site of semen deposition is optimized (Casas et al., 2010; Colenbrander et al., 2000). These techniques involve placing the semen dose directly into the uterus for IUI (Roca et al., 2006b) or in one side of the uterine horn for DIUI (Tummaruk and Tienthai, 2011).

For IUI, a conventional trans-cervical AI catheter is inserted into the cervical folds, and a thin, semi-rigid insemination rod is passed through the conventional catheter, through the cervix, and into the uterine body (Roca et al., 2006b; Watson and Behan, 2002). For DIUI, a catheter is inserted in the same way as the IUI catheter and then inserted deeper into the uterine lumen for semen deposition in the proximal 1/3 of one uterine horn (Roca et al., 2006b). This AI technique shortens the distance to the site of fertilization and enables the use of a lower concentration of sperm than traditional AI (Casas et al., 2010). Moreover, a recent IUI trial involving a total of 9197 sows showed high fertility rates when only 750 million sperm were used at the correct interval prior to ovulation (Roca et al., 2011). The loss of sperm numbers due to backflow has great importance when low sperm numbers are used, but a reduction in the sperm loss during AI is reported to be one of the advantages of IUI and DIUI (Mezalira et al., 2005).

An experiment by Watson and Behan (2002) showed that it was not detrimental to fertility to use lower sperm numbers when inseminating directly into the uterine body (IUI) compared to trans-cervical insemination, and doses as low as 1 billion sperm were sufficient for achieving high fertility regardless of production system, season, boar, sow genotype, or parity. These lower doses allow for more efficient use of semen from genetically superior boars (Tummaruk et al., 2007). Additionally, further reductions in sperm numbers can be achieved when using DIUI, as the sperm are able to bypass phagocytosis in the uterine body. It has been reported that pregnancy rates, farrowing rates, and litter size obtained when inseminating 50 million spermatozoa in 10 mL per sow when using DIUI were not significantly different from those achieved after standard AI (Martinez et al., 2002). In another study, doses with 150 million sperm were shown to result in reduced litter sizes as a consequence of unilateral fertilization (Roca et al., 2006b). This can be averted if the number of spermatozoa inseminated is increased to 600 million, which leads to fertilization in both oviducts (Tummaruk et al., 2007). Additionally, sperm numbers can be decreased further (approximately 10 million cells per dose) when sperm are introduced directly into the oviduct via surgical methods (Colenbrander et al., 2000); however, this procedure is typically reserved for research settings. Overall, the use of IUI and DIUI techniques might be of great benefit to the pig industry because the number of doses per year obtained from a genetically superior boar could be increased from the current recommended 2,000 to at least 40,000 (Martinez et al., 2002; Mezalira et al., 2005).

3.3 Computer Assisted Semen Analysis and Sex-Sorted Sperm

Computer assisted semen analysis (CASA) machines are capable of image analysis with a phase-contrast microscope and computer measurements of motion parameters as a tool for semen evaluation using motion characteristics, morphology, and concentration (Didion, 2008). CASA has been used at the commercial level due to its speed, ability to identify sperm motion parameters and morphology that may be correlated with boar fertility. It is thought that the use of CASA could lead to identification of sub-fertile ejaculates rather than predicting boars with high fertility (Didion, 2008).The development of semen sexing technology has great potential for pre-selection of sex in swine (García et al., 2007). However, sperm are sensitive to the different steps of the sexing process involving flow cytometry that include staining, high dilution, and laser exposure (Maxwell et al., 1998) which damages sperm and results in a very short fertile lifespan (García et al., 2007).

3.4 Ovulation Induction Procedures

Since ovulation time in gilts and sows has been known to vary between 10 and 85 h after the onset of estrus (Kemp et al., 1998), the desire to lower the number of sperm needed in the insemination dose has led to the development and use of ovulation induction hormones. These ovulation induction drugs would be beneficial as they would allow for single fixed-time inseminations, which would lead to better utilization of semen from genetically superior boars (Foxcroft et al., 2008). They could also decrease the need for estrus detection. Synchronized ovulation has the potential for insemination of all females at approximately the same time during

the peak time of fertility. Different hormones tested such as pLH or hCG induce ovulation between 36 and 38 h (Abad et al., 2007a; Garcia et al., 2007) and 40 and 42 h (Bertani et al., 1997; Hunter and Dziuk, 1968), respectively. While an injection of PG 600 (400 IU eCG + 200 IU hCG) has been shown to increase the ovulation rate, litter size does not increase (Breen et al., 2006). It was hypothesized that this is due to the fact that PG 600 may alter the timing of ovulation, such that breeding at 12 and 24 h may not be optimal (Horsley et al., 2005). Knox et al. (2011) showed that using intravaginal triptorelin (TG-96), a GnRH agonist, 96 h after weaning advances ovulation in order to facilitate fixed-time insemination. With all of these ovulation induction drugs, the follicular status of the sow or gilt is vital since follicles must be present that are mature enough to respond to ovulation induction (Knox et al., 2011).

Combining these advanced technologies could lead to high fertility using lower sperm numbers or less fertile sperm like FTS for AI. CASA machines could be used to predict potential fertility of a given sample, and combining induced ovulation with IUI or DIUI procedures provides an opportunity to deposit the lowest number of sperm at the optimum time prior to ovulation necessary to achieve acceptable fertility.

3.5Artificial Insemination using Frozen-Thawed Boar Sperm

Cryopreserved boar sperm is used in less than 1% of all commercial inseminations (Johnson et al., 2000) due to the decrease in pregnancy rate and litter size in comparison to liquid extended semen (Didion and Schoenbeck, 1995). The main reason for this is the poor survival rate of the spermatozoa (Cerolini et al., 2001), which consequently results in fewer doses produced and a greater concentration needed in the insemination dose. Data suggests a reduction

in the number of doses by 30-40% when compared to AI with liquid semen (Almlid et al., 1987). Other limitations to FTS include higher investment costs for lab equipment, supplies, and labor.

3.5.1 Variation in Freezing Ability Between Boars

Individual boars have been shown to have variability in the freezing ability of their ejaculates over time, and under a fixed procedure, the individual boar, and not the breed determined the survival rate after freezing (Thurston et al., 2002; Waterhouse et al., 2006). This was confirmed by Roca et al. (2006a), who reported that despite rigorous selection criteria of sires prior to freezing, there was considerable variability of sperm cryo-surival between ejaculates, especially between boars. This study showed that boar accounts for more than 70% of the total variance among ejaculates in post-thaw sperm quality and while the reason for the variability is unknown, it is thought to have a genetic origin.

The terms "good freezer" and "bad freezer" have existed for a long time, however, classification as a "good freezer" does not guarantee a high fertilizing ability for frozen sperm in terms of *in-vitro* and *in-vivo* fertility (Cremades et al., 2005). Even though the selection of "good freezers" could have a tremendous impact on the success of boar sperm cryopreservation (Peña et al., 2007), there is no conclusive assay to predict "good freezing" boars. Boars showing good freezability could maintain this condition over time, which suggests that assessment of a single ejaculate after cryopreservation may be sufficient to identify those as good or poor freezers. Between 20 to 33% of stud sires are considered "poor" freezers, which is most likely genetically driven, and the ability to cryopreserve these ejaculates remains important for genetic resource banking and international exchange of genetic material (Rath et al., 2009). Those males with

high pre-freeze fertility may not have high post-thaw fertility, and vice versa. This appears to be a result of individual differences in the composition of the sperm plasma membrane (e.g. cholesterol:phospholipid ratio and phospholipid:protein ratio), and the distribution of the membrane components which influence semen freezability (Holt, 2000b; Zeng et al., 2001). However, the main deterrent against widespread use of AI with frozen-thawed boar sperm is reduced pregnancy rates (Garcia et al., 2010), a decrease in farrowing rate by 20-30% (Abad et al., 2007a; Bertani et al., 1997; Johnson et al., 2000), and a reduction in litter size by 2-3 piglets on average (Bailey et al., 2008; Bertani et al., 1997; Johnson et al., 1981).

3.5.2 Frozen-Thawed Sperm in the Female Reproductive Tract

Boar sperm cooling followed by cryopreservation decreases the fertilizing capacity of the spermatozoa (Bailey et al., 2008; Pelaez et al., 2006) due to not only a decrease in motility and viability, but also due to the subtle changes in sperm membrane structure and function (Colenbrander et al., 2000). Over 90% of inseminated spermatozoa are eliminated from the female reproductive tract within 2-3 h after insemination due to semen back flow and phagocytosis. This loss is highest when the spermatozoa are weak or damaged as is the case with frozen-thawed sperm (Roca et al., 2006b) because damaged sperm are more vulnerable to backflow and phagocytosis while in the female reproductive tract. Due to this vulnerability, few FTS are able to pass through the cervical folds and establish an adequate sperm reservoir, which may partly explain the decreased fertility (Roca et al., 2006b). Increasing the number of FTS in the insemination dose to ~5 billion (Saravia et al., 2005) can partially compensate for this

decrease in numbers of sperm, however, this increase significantly decreases the efficiency for use of each ejaculate.

3.5.3 AI Timing with Frozen-Thawed Sperm

Soede et al. (1995b) showed that the percentage of normal embryos tended to decline when a single insemination with liquid semen took place greater than 24 h prior to ovulation. However, when a second insemination was administered within the 24 h period before ovulation, the number of normal embryos increased. This suggests that fresh semen can survive in the female reproductive tract for up to 24 h. In contrast, when frozen semen was used, Waberski et al. (1994) showed that gilts inseminated from 4 to 0 h prior to ovulation had much higher pregnancy rates than gilts inseminated greater than 8 h prior to ovulation and suggests that cryopreserved sperm have a much shorter fertile life-span in the female reproductive tract when compared to liquid semen (Watson, 2000).

3.5.4 Benefits of Frozen-Thawed Sperm

Despite its current fertility limitations, cryopreserved boar sperm would aid in biosecurity and protection of breeding herds from pathogens transported through semen. Storage of sperm in frozen form would allow for sufficient time for disease testing in sires (Bailey et al., 2008; Eriksson, 2000; Garcia et al., 2010) and would also offer an effective means for preserving and disseminating superior genetic material over great distances and time periods (Okazaki et al., 2009; Roca et al., 2006b). Sperm in frozen form can be transported across long distances or held

until the female is in estrus (Bailey et al., 2008) without any change in fertility while in storage or transit. It may also help to provide for a supply of germplasm in the case of natural disasters (Didion and Schoenbeck, 1995), disease outbreak, or adverse climactic conditions (Cerolini et al., 2001). A stock of frozen semen is practical for transport of gametes for international markets (Bailey et al., 2008) and enables the re-establishment of certain genetics even after the sire has died (Bailey et al., 2000). While fresh semen has the potential for storage for 7-10 days (Vyt et al., 2004a), FTS has the potential to be stored indefinitely. Recent improvements in cryopreservation protocols, such as alternative packaging systems (Eriksson et al., 2001) or the optimization of centrifugation during processing (Carvajal et al., 2004) have contributed to better post-thaw sperm viability, which has been one of the drawbacks of cryopreservation in the pig.

3.5.5 Cryopreservation Packaging Systems

Advancements in FTS packaging systems have included the use of 0.25 – 5.0 mL plastic straws and FlatPacks. Due to the large volume needed for successful porcine AI, 5 mL straws were the most widely used packages (Almlid and Hofmo, 1996). However, due to the large surface area-to-volume ratio, too rapid cooling in the periphery and too slow cooling in the center lead to a greater potential for acrosome and membrane damage. This damage could be reduced by smaller straw volumes (Bwanga et al., 1990; Weitze et al., 1987). In general, packaging systems with a larger surface area to volume ratio, such as pellets, 0.25 - 0.5 mL straws, and FlatPacks, allow a more homogeneous freezing and thawing temperature throughout the sample (Eriksson, 2000).

4. Cryopreservation of Boar Sperm

4.1Freezing and Thawing Methods

Several studies have identified an optimal pre-freeze holding temperature and cooling rate for boar sperm. It is important to control the temperature and conditions of the samples from the time of collection, through dilution and storage, cooling and cryopreservation, thawing, and prior to insemination because even the slightest shifts in temperature can dramatically affect sample quality. An experiment by Purdy et al. (2010) found that the optimal pre-freeze holding temperature is 16°C. This temperature must be maintained during pre-freeze storage or decreases in total and progressive motility will occur. This study demonstrated that it is important to properly dilute and cool boar semen, regardless of whether the samples will be used fresh or frozen. Samples that are not properly treated can induce capacitation and the acrosome reaction. Medrano et al. (2009) and Hernandez et al. (2007) showed that the effects of cooling rate on sperm cryosurvival are dependent on individual boar differences. This suggests that sperm freezability depends on more intrinsic sperm properties, like plasma membrane composition and other genetically determined characteristics than on the freeze-thaw process alone. It was also shown that cooling rate affected sperm cryosurvival in different ways from different boars. Some boars were more susceptible than others, but cryosurvival was generally better when cooling was done at faster rates. However, if cooling rates are too fast, water is unable to diffuse out of the spermatozoa into the surrounding medium, and the cell will cool to a point where lethal intracellular freezing takes place. If cooling is too slow, water passes out of the spermatozoa, effectively preventing intracellular ice formation, but leaves solutes within the cell at an elevated

concentration. Changes in the pH and ionic composition of the extracellular environment can induce disruption of the sperm plasma membrane (Songsasen and Leibo, 1997) and may even cause DNA denaturation (Hammadeh et al., 1999). When boar sperm are subjected to freezing temperatures, extensive damage occurs between 0 and -20 °C, a range of temperatures that has to be crossed twice during the freezing and thawing processes (Bwanga, 1991). This temperature range is known as a transition period, and damage in this period has been attributed to the formation of small intracellular ice crystals in some cells which grow during a slow rewarming process (Holt, 2000a). As a majority of cellular damage occurs during this period, it is imperative that fluctuations in cooling rate are kept to a minimum. Unfortunately, the increase in temperature caused by ice formation and the resultant release of the latent heat of fusion means that the sperm is effectively cooled to -5 °C but is then exposed to a temperature increase of up to 7 °C, resulting in extensive cellular damage (Thurston et al., 2003). Because of this, sperm samples benefit from a fast temperature change and fast heat transfer throughout the sample. However, while use of a higher thaw temperature (such as 70°C or higher) would provide a faster warming rate (Aamdal and Andersen, 1968; Hernandez et al., 2007; Pursel and Johnson, 1975), the time of straw removal would become progressively more critical (Hernandez et al., 2007; Pursel and Park, 1987).

4.2 Additives to cooling and freezing media

After ejaculation, the spermatozoa are mixed with seminal plasma. Seminal plasma supplies the sperm with the necessary nutrients for the high metabolic demands of transport through the female genital tract. The high metabolic activity can only be maintained over a

limited period (Gadea, 2003). In order to preserve sperm quality after thawing, the metabolic activity of the sperm must be reduced. This can be accomplished through the use of additives included in freezing media to protect the sperm such as antioxidants, antimicrobials, surfactants, and cryoprotectants. A chelating agent (EDTA) is added to semen extenders to block the action of calcium as a mediator of sperm capacitation and the acrosome reaction (Gadea, 2003). Antibiotics are necessary additions to the extender since the components, such as glucose, and the temperature at which fresh semen doses are stored (15 – 16 °C) promote the growth of most gram negative bacteria which can be shed through the testes, other tissues of the genital tract, or preputial fluids (Gadea, 2003). Sperm are exposed to greater oxidative stress during the cooling, freezing, and thawing processes, and it has been shown that sperm quality is improved when vitamin E and ascorbate are added to the media. These natural antioxidants have protective effects on metabolic activity and cellular viability of sperm (Bailey et al., 2000; Bathgate, 2011). Antibiotics can be introduced into the ejaculate through dietary supplementation with selenium to increase levels in the seminal plasma which will lead to protection of the sperm cells during processing, or through direct addition to the extender used in the freeze-thaw process (Bathgate, 2011). Certain pathogens are commonly transmitted through the semen, which is a reason that antimicrobials like aminoglycosides, B-lactams, and lincosamides are added to suppress pathogens and aid in biosecurity practices (Maes et al., 2008). Bacterial contamination in the sample can lead to a series of alterations including diminished sperm motility, agglutination, increased proportion of altered acrosomes, and pH lowering to acidic levels (Gadea, 2003). Most extenders used for freezing boar semen, including Beltsville Thawing Solution (BTS), are based on egg yolk and glycerol as cryoprotecting agents and a high concentration of sugars and a detergent (Gadea, 2003), although in certain instances dimethyl sulphoxide (DMSO) may be a

better cryoprotectant (Holt, 2000a). Unfortunately, glycerol is cytotoxic at body temperature, which means boar sperm must be exposed at ~5°C, which slows permeation into the cell (Rath et al., 2009). The addition of glycerol alters the dynamics of specific areas of the sperm plasma membrane (Buhr et al., 2001), prevents some of the phase transitions during cooling by increasing the water permeability and fluidity of sperm plasma membranes (Bailey et al., 2000), and improves cryosurvival in those boars considered "moderate" or "poor" freezers (Rath et al., 2009). Arguably the most commonly used short-term semen extender in the world, BTS is further characterized by containing a small amount of potassium which preserves the sodium-potassium pump and thus avoids intracellular potassium depletion, which is related to reduced sperm motility (Gadea, 2003). In contrast, Androhep Cryoguard Extender is a commonly used long-term extender and is further comprised of Hepes as a pH regulator and BSA to compensate for the dilution effect on seminal plasma proteins (Gadea, 2003).

4.3 Damage Associated with Cryopreservation

4.3.1 Membrane Damage

There are many opportunities for sperm damage under the conditions of cryopreservation. The cells are exposed to major osmotic pressure changes which can lead to hypotonic shock (Petrunkina et al., 2007), ice crystal formation (Watson, 2000), and cryocapacitation (Abad et al., 2007a), but the most severe damage to sperm occurs in the plasma membrane (Bailey et al., 2008; Cremades et al., 2005; Rath et al., 2009). Damage appears to be related to membrane oxidation due to the increased production of reactive oxygen species (ROS) that occurs during the freezing and thawing processes (Bathgate, 2011). The plasma membrane of the sperm head

isolated from frozen-thawed sperm cells are less dynamic than fresh sperm, and often show ultrastructural damage, morphological defects, phosphorylation patterns, in addition to missing or abnormal acrosomes (Bailey et al., 2000). Membrane damage can also affect motility and viability of the sperm cells. Cremades et al. (2005) observed that while the percentages of viable and motile sperm were high (> 50%) prior to freezing, both of these measures fell drastically after freezing and thawing. Boar sperm membranes are more sensitive to cold shock than those of other mammals because they have a lower cholesterol:phospholipid ratio, which can cause greater susceptibility to damage earlier in the cooling process (Bailey et al., 2008). Each ejaculate appears to contain a heterogenous mix of sperm cells in how they react to osmotic stress. The proportion of cells that survive the cryopreservation process is partially determined by the sensitivity to osmotic stress during freezing and thawing (Watson, 2000). Cold shock irreversibly causes sperm motility and metabolic activity to decrease, as well as destroying the selective permeability of the sperm membranes which can lead to cell death (Bailey et al., 2000). Specifically, this sensitivity to cold seems to be related to the lipid content of the sperm cell membranes; thus, when the temperature falls, lateral movements of membrane phospholipids are reduced and this causes separation of the lipid phases, which is associated with irreversible alterations to membrane proteins (Gadea, 2003).

4.3.2 Cryocapacitation

The membrane changes of frozen-thawed sperm are very similar to those of fresh sperm that are in advanced stages of capacitation, a phenomenon known as cryo-capactiation (Rath et al., 2009). Cryo-capacitation, which is the induction of a capacitation-like status of sperm cells

induced through the freezing process, is often a cause of a decrease in conception and farrowing rates (Bailey et al., 2003). After ejaculation and deposition in the female reproductive tract, the sperm have to go through an activation process to be capable of fertilizing an egg. This process is known as "capacitation," which is defined by the functional changes usually taking place in the oviduct of the female (Petrunkina et al., 2007). The sperm must capacitate in order to react to the zona pellucida of the oocyte, induce the acrosome reaction, and finally fertilize the oocyte (Topfer-Petersen et al., 2002). When this process happens too quickly, as is the case with cryo-capacitation, the sperm are incapable of forming a functional sperm reservoir in the oviduct resulting in a greatly reduced fertile lifespan (Abad et al., 2007b), lowering the number of available sperm cells, and decreasing the chances for successful fertilization.

4.3.3 Ice crystal formation

To minimize ice crystal formation for optimal cell survival, the freezing rate should be fast, but has to be slow enough to allow the intracellular water to exit the cell, preventing ice formation within the cell (Watson, 2000). When cells are frozen they are subjected to stresses resulting from the water-solute interactions that arise through ice crystallization. Exposure of cells to the hyperosmotic, yet unfrozen solution, causes withdrawal of intracellular water, consequent cell shrinkage, and influx of ions. Thawing involves a reversal of these effects, and the consequent inward water influx may cause cell membrane disruption (Holt, 2000a). An increase in the thawing rate has been shown to reduce re-crystallization of intracellular ice, a process that can lead to the formation of larger and more stable ice crystals that can damage the cells (Fiser and Fairfull, 1990). On the other hand, rapid warming can induce osmotic stress on

the spermatozoa because of the abrupt melting of the extracellular solution that can cause unbalanced rates of water influx and cryoprotectant exit from the cell. This can lead to swelling and lysis of the cells (Hammerstedt et al., 1978; Mazur, 1984). The warming rate appears to be important at two phases in the warming cycle, one below freezing (to prevent re-crystallization of ice crystals) and one above freezing. While a high warming rate is beneficial during thawing, especially below freezing, it was detrimental to sperm survival after samples had warmed to 20 °C or higher before removal from the waterbath (Pursel and Park, 1987). Fiser and Fairfull (1990) found that the percentage of motile sperm and sperm with a normal apical ridge was greater with faster thawing rates for semen frozen in 0.5 mL straws. However, Fiser et al. (1993) proposed that boar sperm may be damaged critically by slow cooling alone, and only a limited "rescue" could be achieved by rapid thawing.

4.4 Evaluation and prediction of ejaculate fertility

4.4.1 Motility evaluation

Laboratory assessment of sperm quality is an essential procedure for assisted reproduction in domestic species, as it helps to improve the selection of males with good reproductive performance (Gadea, 2005). The choice of parameters to assess must be reproducible, fast, and sensitive enough to detect fertility differences. Measures such as motility, morphology, and concentration appear insufficient to detect differences between good and moderate ejaculates (Foxcroft et al., 2008; Petrunkina et al., 2007; Woelders, 1991) and can only detect those samples with markedly poor quality (Gadea, 2005). More recently, considerable

attention has been given to the study of oocyte-sperm interactions (Gadea, 2005) and sperm characteristics that pertain to the way the sperm will function in the female reproductive tract. These characteristics include the ability to pass through cervical mucus (Maxwell et al., 2000), escape phagocytosis by uterine leukocytes, pass the utero-tubal junction (UTJ) and form a functional sperm reservoir, as well as their ability to capacitate and respond to changes in the female tract (Colenbrander et al., 2000).

Tests of fertility in the male include motility estimates, metabolic measures, morphology, *in-vitro* penetration of oocytes, non-return rates, conception rates, and litter size. Conception rates, the proportion of inseminated females farrowing, and litter size are considered the ultimate measures of sperm quality and fertility. However, these measures can prove inaccurate due to variation in the fertility of females, seasonal variation, differences in breeding herd management practices, human error, and lack of uniformity (Dziuk, 1996). Semen analysis alone is not necessarily a good predictor of either fertility or infertility (Waberski et al., 2008). Popwell and Flowers (2004) concluded that common semen quality traits, such as motility, head and tail morphology, and acrosome morphology have limited value in terms of relative *in-vivo* fertility of ejaculates. In contrast, progressive motility has been positively correlated with the number of pigs born alive (Gadea, 2005). Gil et al. (2005) found that while post-thaw boar sperm quality can separate "good" ejaculates from "bad" ejaculates which can relate to *in-vitro* or *in-vivo* fertility, there are ejaculates outside this pattern. A relatively high number of characteristics from both the male and female side affect the chance of successful fertilization and gestation. From the boar side, specific ejaculate, handling, storage, season, and technicians have great effects on fertility, and on the female side, estrus duration, farm management, parity, and season have their influence. Once inside the female reproductive tract, the uterine and oviductal environment,

ovulation time, oocyte quality, sperm capable of capacitation and undergoing the acrosome reaction, and oocyte penetration at the site of fertilization must occur (Daas, 1992). Yet the ultimate measures of boar fertility are pregnancy rate and number of pigs born alive (Foxcroft et al., 2008).

Motility is a well established parameter for excluding ejaculates from semen processing, freezing, and insemination; however, it must be considered in two ways. Firstly, the proportion of cells which exhibit motility, and secondly, the quality of the motility itself (Holt, 1995). That is, the speed and direction of movement should be measured. The given threshold for exclusion is usually lower than 70% motility (Waberski et al., 2008). Motility evaluations can be conducted subjectively using a light or phase contrast microscope or objectively using Computer Assisted Semen Analysis (CASA) machines. It was reported by Vyt et al. (2004b) that repeatability of suboptimal motility measurements are more difficult when using the subjective method. With recent advancements, CASA systems yield much more precise results with greater repeatability of samples, as well as more detailed information on sperm cell movements. Using CASA, total sperm motility has been shown to be positively correlated with total litter size (Sutkeviciene et al., 2009).

A study by Cerolini et al. (2001) confirmed that traditional sperm quality parameters, like motility and viability, were significantly decreased after the freezing and thawing procedures by approximately 50% (Almlid et al., 1987). The decrease in sperm quality depended on the quality of the ejaculate prior to freezing. In this case, lower quality ejaculates had a tendency to be more affected by sub-optimal freezing and thawing procedures than higher quality ejaculates. In a study by Cerolini et al. (2001), both motility and viability were significantly decreased after the freeze-thaw process. The magnitude of loss of sperm quality differed according to the pre-freeze

quality measures in that initial poor quality semen was more affected. It was shown that high sperm viability prior to freezing is indicative of high motility and viability after freezing and thawing. Conversely, Roca et al. (2006a) reported that increased sperm concentration, together with the greatest proportion of sperm with normal morphology and motility before freezing did not necessarily guarantee high sperm survival after the freezing and thawing processes. Moreover, ejaculates with low sperm concentration and moderate sperm quality prior to the freeze-thaw procedures could have good sperm quality post-thaw.

4.4.2 Fluorescent staining

Fluorescent staining allows researchers to visualize the integrity of the membrane and the status of the acrosome, as well as other functional aspects of the sperm cells. The plasma membrane is responsible for the preservation of cellular homeostasis and plays a vital role on sperm survival inside the female reproductive tract and on preservation of sperm fertilizing capacity (Andrade et al., 2007; Flesch and Gadella, 2000). Because of the inability of sperm cells to repair the cell membrane when damage occurs, an intact membrane is essential for fertility (Daas, 1992). Plasma membrane integrity can be assessed by flow cytometry using membrane-impermeable DNA-binding dyes such as propidium iodide (PI), SYBR-14, and Yo-Pro-1. Propidium iodide has become a useful counter stain for many types of tests, as it only penetrates damaged nuclear membranes and intercalates with the DNA, which leads to positive identification of dead cells (Maxwell et al., 2000). When stained by the SYBR-14/PI technique, the intact cell nuclei appear green since SYBR-14 binds to chromatin, but nuclei fluoresce red once membrane damage has occurred (Holt, 2000a). Acrosome integrity can be assessed

simultaneously by using impermeable fluorochrome-conjugated peanut agglutinin (FITC-PNA) (Waberski et al., 2008). The acrosome is essential for the function of the sperm cell. After the acrosome reaction, the enzyme contents of the lysosome are exposed and the stain can then bind to these contents (Daas, 1992). Cells that bind to the stain have acrosome reacted and cannot fertilize an egg because sperm cannot fuse with the zona pellucida. These staining techniques are able to directly detect membrane changes and their relation to freezability, thus classifying ejaculates prior to freezing as good, moderate, or poor (Peña et al., 2007). However, several studies have demonstrated that the results obtained from sperm viability testing have no significant correlation with fertility *in-vitro* or *in-vivo* (Foxcroft et al., 2008). Perhaps these staining techniques, combined with other quality measurements will be more useful in predicting fertility of ejaculates.

5. Experiment 1: Effect of thawing temperature and duration of thawing on in-vitro fertility measures of boar sperm cryopreserved in 0.5mL straws

5.1 Abstract

Thawing rate has been shown to influence post-thaw quality of frozen-thawed boar sperm (FTS), but there is currently no standard protocol for thawing. The objective of this experiment was to evaluate the effect of thaw temperature and duration of thawing on sperm motility, viability, and the percentage of sperm with intact acrosomes. Ejaculates (n = 15) from 13 boars were frozen in 0.5 mL straws at a concentration of 1.4 x 10^9 cells/mL. To test for the effects of temperature and duration of thawing, individual straws from each ejaculate were assigned to each treatment. The treatments included straws (n=15/treatment) thawed at 50 °C for 10s, 20s, and 30s and at 70°C for 5s, 10s, and 20s. Following thawing, FTS was expelled into glass tubes in a 37°C heating block and evaluated for post-thaw motility (PTM), viability (VIA), and the percentage of live sperm with intact acrosomes (IA). Samples were evaluated at 5, 30, and 60 min for PTM at 200X, for VIA using propidium iodide, and for IA using FITC-PNA staining. Data were analyzed for the effects of temperature, duration of thawing, and boar. There was an effect of temperature and duration of thawing ($P < 0.0001$) on PTM, VIA, and IA. There was no difference in PTM (~41%) across all treatments except when thawing at 70°C for 20s (3%). Thawing for only 5 s at 70 °C resulted in lower VIA (50%) compared to other treatments (~56%), however, thawing at 70°C for 20s resulted in the lowest VIA (6%). All treatments had ~80% IA, except when thawing at 70°C for 20s (62%). The results indicate that when thawing boar sperm in 0.5 mL straws with the cryoprotectant used, 70°C for 20s rendered most sperm

infertile, while thawing at 70°C for 10s and 50°C for 20s resulted in the highest PTM, VIA, and IA.

5.2 Introduction

The use of frozen-thawed boar sperm (FTS) in modern swine production has been limited due to variable pregnancy rates and litter sizes resulting from artificial inseminations using FTS (Hofmo and Grevle, 2000; Johnson, 1985). This is due to a reduced lifespan of FTS both *in vivo* and *in vitro* when compared to liquid extended semen (Einarsson and Viring, 1973; Pursel et al., 1978; Roca et al., 2006b; Waberski et al., 1994). It has been hypothesized that post-thaw handling procedures may have large implications on post-thaw quality measures including motility, viability, capacitation status, and acrosome integrity.

Over the past several decades, novel semen packing systems have been developed to replace the previously used pellets and ampules (Pursel and Johnson, 1975). Currently cryopreserved boar sperm is most commonly packaged in 0.25, 0.5, and 5 mL plastic straws or in plastic bags referred to as MiniFlatPacks and FlatPacks (0.5-0.7 mL and 5 mL) (Bwanga, 1991; Eriksson et al., 2002; Eriksson and Rodriguez-Martinez, 2000; Johnson et al., 2000). While the volume of the straws and plastic bags may be variable, better post-thaw quality was observed when using lower volumes (0.25 mL to 0.7 mL) due to the smaller surface area to volume ratio allowing more equal freezing and thawing (Bwanga, 1991; Cordova et al., 2001; Pelaez et al., 2006). Due to these discrepancies in freezing volume, surface area, and surface area to volume ratio, different thawing temperatures and durations of thawing have been explored depending on the volume of the straw. Early experiments conducted thawing at 37 °C, however, this procedure

was later abandoned in favor of higher thawing temperatures such as 50 °C (Cordova-Izquierdo et al., 2006; Pelaez et al., 2006) and 70 °C (Hernandez et al., 2007). Within these temperature ranges there is a variation in thawing duration based on the volume of straws, with lower volume straws (0.25-0.5 mL) being thawed from 10-20 s at 50°C (Aamdal and Andersen, 1968; Pelaez et al., 2006)and 5-10 s at 70 °C (Hernandez et al., 2007), and higher volume straws (5-7 mL) being thawed for 40 s at 50 °C (Cordova-Izquierdo et al., 2006).

While there has been extensive research on the importance of pre-freeze semen handling and temperature to determine optimal pre-freeze storage temperature and duration (Guthrie and Welch, 2005; Johnson, 1985; Watson, 1995), cooling rate (Pursel et al., 1973; Pursel and Park, 1985), and freezing rate (Almlid et al., 1987; Hernandez et al., 2007) to ensure high quality of ejaculates post-thaw; there is little available information on the importance of post-thaw semen handling and its implications on *in vitro* and *in vivo* fertility. The objective of this study was to determine the effect of thaw temperature and duration of thawing on sperm motility, viability, and the percentage of sperm with intact acrosomes on boar ejaculates frozen in 0.5 mL straws.

5.3Materials and Methods

5.3.1. Semen Collection and Freezing

Ejaculates (n=15) from 13 purebred boars at a commercial boar stud in a continuous collection and freezing rotation were collected from March 2010-March 2011. Semen was collected using the gloved-hand technique and diluted 1:1 with Modena extender (Swine Genetics International, Cambridge, IA) at a ratio of 1:1, cooled to 17°, and shipped overnight to

the University of Illinois (Urbana-Champaign, IL) and processed using the procedures of Ringwelski et al. (2013).

Upon semen arrival, the sample was evaluated for concentration and motility and centrifuged at 800 x *g* and the supernatant was aspirated. The sperm pellet was re-suspended with Androhep CryoGuard Cooling Extender (Minitube of America, Verona, WI) to a concentration of 2.8 x 10^9 sperm/mL and held at 5 °C for 2.5 h before dilution to a final concentration of 1.4 x 10^9 sperm/mL withAndrohep CryoGuard Freezing Extender (Minitube of America, Verona, WI). Straws (0.5 mL) were filled and then placed into the Ice Cube controlled rate freezer (Minitube of America, Verona, WI) using the freezing process described by Spencer et al. (2010) and Ringwelski et al. (2013) and stored in liquid nitrogen.

5.3.2 Semen Thawing and Evaluation

Thawing of straws was carried out in a thermally controlled water bath at 50 °C for 10, 20, and 30 s, as well as 70 °C for 5, 10, and 20 s. Preliminary data showed a reduction in motility and viability when thawing at 37°C for 10 and 20 s (Appendix D), which led to the exclusion of this parameter from the experiment. A total of 90 straws were thawed, (n= 6 straws per ejaculate). Samples were diluted in Androhep CryoGuard Thawing Extender (Minitube of America, Verona, WI) at 1:40 for motility and 1:400 for determining concentration and held in a 37 °C heating block. Evaluation was performed at 5, 30, and 60 min after thawing. Samples were examined under a phase contrast microscope with a 37 °C heated stage at 200X magnification. Ten fields were examined to evaluate 100 sperm/slide and motility was expressed as a percentage of the total number of sperm cells. Fluorescent staining was performed in order to

determine membrane viability using propidium iodide (PI, Sigma Aldrich, St. Louis, MO) and acrosome integrity using Fluorescein isothiocyanate lectin from peanut agglutinin (FITC-PNA, Sigma Aldrich, St. Louis, MO). Samples were thawed and diluted 1:50 in 26 °C Beltsville Thawing Solution® (Minitube of America, Verona, WI) and co-incubated for 10-15 min with the fluorescent stains for 15 min and fixed with 0.4% paraformaldehyde in PBS solution. A total of 300 sperm was analyzed for fluorescence using a Carl Zeiss AxioCamHRc (Carl Zeiss Microscopy, LLC. Thornwood, NY) at 400X magnification and the percentage of PI negative and FITC-PNA positive sperm calculated from the total number of sperm evaluated.

5.4 Statistical Analysis

Data were analyzed using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC). Continuous response measures were analyzed using the PROC MIXED procedures for significance of the main effects using the *F*-test and differences between least squares means identified using the *t* test. All models for the dependent variables included the main effects of treatment, storage time, and boar, and for the interaction between treatment and storage time.

5.5 Results

There was no interaction between treatment and storage time for MOT, VIA, or IA ($P > 0.05$). Motility, viability, and the percentage of live sperm with intact acrosomes was affected by treatment, storage time, and boar ($P < 0.0001$). There was an effect of treatment, with thawing at 70°C for 20s resulting in decreased MOT (3%) compared to all other treatments (40%; Table

5.1). Viability was also reduced when thawing was conducted at 70°C for 5s (50%) and 20s (6%) compared to all other treatments (56%; Table 5.2). There was an effect of treatment on the percent of live sperm with intact acrosomes with thawing at 70°C for 20s resulting in a reduction in IA (62%) when compared to other treatments (86%; Table 5.3).

5.6 Discussion

Results of this study can have large implications on both *in vitro* and *in vivo* fertility of cryopreserved boar sperm. As reported by this experiment, improper thawing processes can greatly affect the post-thaw quality of FTS. Previous studies have shown that thawing at higher temperatures for shorter periods of time resulted in the highest post-thaw quality for cryopreserved boar sperm (Fiser et al., 1993; Hernandez et al., 2007; Muinoa et al., 2008) however, it was unclear as to whether ultra-high temperatures, such as 70 °C, are needed to achieve this increase in quality.

An increase in the thawing rate has been shown to reduce re-crystallization of intracellular ice, a process that can lead to the formation of larger and more stable ice crystals that could damage the cells (Fiser and Fairfull, 1990). On the other hand, rapid warming can induce osmotic stress on the spermatozoa because of the abrupt melting of the extracellular solution. This can cause unbalanced rates of water influx and cryoprotectant exit from the cell and lead to swelling and lysis of the cells (Hammerstedt et al., 1978; Mazur, 1984). Also, while use of a higher thaw temperature, such as 70 °C or higher, would provide a faster warming rate that may be beneficial for thawing (Aamdal and Andersen, 1968; Hernandez et al., 2007; Pursel and Johnson, 1975), the time of straw removal would become progressively more critical (Hernandez

et al., 2007; Pursel and Park, 1987) as ultra-high temperatures can cause proteins in the sperm membrane to denature. This could help explain why thawing at 70 °C for 20 s had such a detrimental effect on the sperm.

In this study, it was found that thawing 0.5 mL plastic straws of cryopreserved boar sperm at 70 °C for 10 s yielded no difference in any measures of post-thaw quality from 0.5 mL straws thawed at 50 °C for 10, 20, or 30 s. However, thawing for just a few seconds shorter or longer than 10 s can have extremely detrimental effects. Based on the results of this experiment, we suggest thawing at 50 °C for 20 s as it provides optimal fertility as well as providing some flexibility in thawing duration.

5.7 Tables

Table 5.1
Least squares means for post-thaw motility of frozen thawed boar sperm in response to the effect of thawing temperature and duration of thawing assessed at specific time intervals.

		Storage time after thawing		
Treatment[c]	n[d]	5 minutes	30 minutes	60 minutes
50°C 10s	15	$43.8^{a,x} \pm 1.3$	$41.9^{ab,x} \pm 1.0$	$40.0^{b,x} \pm 1.3$
50°C 20s	15	$44.3^{a,x} \pm 1.1$	$40.4^{b,x} \pm 0.8$	$39.0^{b,x} \pm 1.1$
50°C 30s	15	$41.9^{a,x} \pm 1.5$	$40.6^{ab,x} \pm 1.1$	$38.8^{b,x} \pm 1.3$
70°C 5s	15	$42.3^{a,x} \pm 1.0$	$39.7^{a,x} \pm 1.0$	$39.4^{a,x} \pm 0.9$
70°C 10s	15	$44.6^{a,x} \pm 1.2$	$41.9^{ab,x} \pm 1.1$	$40.1^{b,x} \pm 1.3$
70°C 20s	15	$4.1^{b,y} \pm 1.1$	$3.7^{a,y} \pm 0.9$	$2.3^{a,y} \pm 0.7$
P-value		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[(a,b)] Within a row, means without a common superscript are different ($P < 0.05$).
[(x,y)] Within a column, means without a common superscript are different ($P < 0.0001$).
[c]There was a main effect of treatment and storage time, but no interaction ($P = 0.7228$).
[d]Number of straws. Straws were from different sires and ejaculates and were equally represented across all treatments.

Table 5.2
Least squares means for post-thaw viability of frozen thawed boar sperm in response to the effect of thawing temperature and duration of thawing assessed at specific time intervals

Treatment[a]	n[b]	Storage time after thawing		
		5 minutes	30 minutes	60 minutes
50°C 10s	15	$55.8^{x} \pm 1.7$	$55.3^{x} \pm 2.8$	$55.1^{x} \pm 2.0$
50°C 20s	15	$57.2^{x} \pm 2.5$	$56.7^{x} \pm 1.5$	$54.3^{x} \pm 1.9$
50°C 30s	15	$53.7^{xy} \pm 2.7$	$54.9^{xy} \pm 2.4$	$52.9^{xy} \pm 2.5$
70°C 5s	15	$50.5^{y} \pm 2.8$	$50.1^{y} \pm 2.6$	$50.1^{y} \pm 3.0$
70°C 10s	15	$57.5^{x} \pm 2.0$	$59.7^{x} \pm 2.5$	$56.1^{x} \pm 2.6$
70°C 20s	15	$5.0^{z} \pm 0.9$	$5.6^{z} \pm 1.7$	$6.9^{z} \pm 1.7$
P-value		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

$^{(x,y,z)}$ Within a column, means without a common superscript are different ($P < 0.0001$).

[a]There was a main effect of treatment and storage time, but no interaction ($P = 0.9792$).

[b]Number of straws. Straws were from different sires and ejaculates and were equally represented across all treatments.

Table 5.3
Least squares means for the percentage of live sperm with intact acrosomes of frozen thawed boar sperm in response to the effect of thawing temperature and duration of thawing assessed at specific time intervals

Treatment[c]	n[d]	Storage time after thawing		
		5 minutes	30 minutes	60 minutes
50°C 10s	15	$84.3^{a,x} \pm 1.1$	$80.9^{ab,x} \pm 1.8$	$77.4^{b,x} \pm 2.1$
50°C 20s	15	$84.2^{a,x} \pm 1.3$	$78.3^{ab,x} \pm 1.5$	$76.7^{b,x} \pm 2.2$
50°C 30s	15	$81.9^{a,x} \pm 1.7$	$78.2^{a,x} \pm 2.1$	$77.5^{a,x} \pm 1.8$
70°C 5s	15	$83.2^{a,x} \pm 1.2$	$78.2^{ab,x} \pm 2.1$	$77.5^{b,x} \pm 1.9$
70°C 10s	15	$85.4^{a,x} \pm 1.1$	$79.3^{ab,x} \pm 1.7$	$77.3^{b,x} \pm 2.3$
70°C 20s	15	$65.7^{a,y} \pm 2.8$	$61.6^{ab,y} \pm 3.7$	$58.4^{b,y} \pm 2.8$
P-value		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[(a,b)] Within a row, means without a common superscript are different ($P < 0.05$).
[(x,y)] Within a column, means without a common superscript are different ($P < 0.0001$).
[c]There was a main effect of treatment and storage time, but no interaction ($P = 0.9509$).
[d]Number of straws. Straws were from different sires and ejaculates and were equally represented across all treatments.

6. Experiment 2: The effect of using frozen-thawed boar sperm differing in post-thaw motility in the 1st and 2nd inseminations on pregnancy establishment, litter size and fetal paternity in relation to time of ovulation

6.1 Abstract

Frozen-thawed boar sperm (FTS) could be useful for dissemination of superior genetics to females across great distances and for precision AI. However, FTS has decreased fertility compared to fresh extended semen. Ejaculates from 38 boars were frozen in 0.5 mL straws, and this experiment tested the effect of post-thaw motility (poor (P), 20.2 ± 3.4%; moderate (M), 31.3 ± 4.2%; or good (G), 43.5 ± 3.2%; mean ± SD) on pregnancy rate, litter size, and fetal paternity. Mature synchronized gilts were checked for estrus at 12 h intervals (n = 207). Gilts were assigned at onset of estrus to: 1) first AI with P and second AI with M (P-M); 2) first AI with M and second AI with P (M-P); 3) first AI with G and second AI with M (G-M); and 4) first AI with M and second AI with G (M-G). For each treatment combination, a set of three boars were randomly selected within motility class for their allelic distinction with M sperm from a single boar represented across all treatments and sires used in both 1st and 2nd inseminations. Insemination occurred at 24 and 36 h after onset of estrus. Number of motile FTS in each dose was $0.8 \times 10^9 \pm 0.2$ for P, $1.2 \times 10^9 \pm 0.2$ for M, and $1.7 \times 10^9 \pm 0.1$ for G in 80 mL of extender. Ovulation was determined by ultrasound at 12 h intervals. Pregnancy rate and litter size were assessed at ~ d 32. Data were analyzed for the main effects of treatment, replicate, and insemination to ovulation interval (IOI). Treatment did not interact with IOI ($P > 0.10$) and did not affect ($P > 0.10$) pregnancy rate (57, 67, 71, 76 ± 7.2%, pooled SEM) or total number of

fetuses (9.2, 9.1, 9.5, 10.0 ± 0.8) for P-M, M-P, G-M, and M-G treatments, respectively. Treatment did affect ($P < 0.05$) the number of fetuses sired from the 1st AI (3.1, 7.2, 6.4, 6.3 ± 1.2) and 2nd AI (5.7, 2.6, 3.0, 3.6 ± 0.9) for the P-M, M-P, G-M, and M-G treatments, respectively. The IOI also influenced ($P < 0.05$) the proportion of offspring sired by the 2nd AI (30.0, 57.7, 51.3, 18.3, ± 6.5%), as well as the number of fetuses sired by each AI. The results of this study indicate that classification of FTS for motility had no effect on early pregnancy rate or number of normal fetuses, but did affect the number of fetuses sired from the first and second AI. Although not significant, there appeared to be a reduced pregnancy rate in the P-M treatment, which could suggest that sperm numbers were a limiting factor.

Keywords: AI, fertility, frozen boar sperm, ovulation, paternity, swine

6.2 Introduction

Artificial insemination (AI) is in widespread use in the swine industry as it accounts for greater than 90% of inseminations performed in the United States (Johnson et al., 2000; Weitze, 2000), and it has arguably been the most important management tool leading to improved herd productivity (Bailey et al., 2008). However, because of its reduced fertility when compared to liquid extended semen, FTS is used in less than 1% of inseminations (Johnson et al., 2000). Further, billions instead of millions of sperm are required with fresh or FTS in order to produce acceptable litter sizes (Reicks and Levis, 2008; Watson and Behan, 2002) with multiple inseminations needed to compensate for variation in time of ovulation and to establish pregnancy (Lamberson and Safranski, 2000; Soede and Kemp, 1997). This decrease in fertility is attributed to a shorter fertile lifespan of FTS *in vivo* when compared to liquid extended semen (Waberski et al., 1994; Watson, 2000) due to damage induced during the freeze-thaw process that reduces motility and viability (Bailey et al., 2008; Colenbrander et al., 2000; Pelaez et al., 2006). Despite these limits, commercial applications (Didion et al., 2013; Knox, 2011) could provide opportunities to improve testing, storage and dissemination of semen from valued sires (Bailey et al., 2008).

Motility is an important indicator for liquid semen use (Gadea, 2005), and >70% motility is the industry standard since a reduction in fertility is seen when used below this measure (Flowers, 1997; Gadea et al., 2004). However, most FTS falls below 60% motility (Medrano et al., 2009; Purdy, 2008) with variation among (Hernandez et al., 2007) and within boars (Pelaez et al., 2006). Motility of FTS affects *in-vitro* (Gil et al., 2008) and *in-vivo* (Casas et al., 2010) measures of fertility, but there is no information on FTS motility in relation to interval from

insemination to ovulation in a multiple AI system. This information could be important for predicting fertility of FTS with different quality, timing inseminations, and changing requirements for number of sperm.

Previous field fertility trials using FTS have used different post-thaw motility cut-off values ranging from >30% total motility (Spencer et al., 2010), to >40% (Ringwelski et al., 2013) and >50% (Didion et al., 2013). There is currently no conclusive evidence to predict the effect of post-thaw motility on pregnancy rate and litter size (Peña et al., 2007) as those males with high pre-freeze motility may not have high post-thaw motility (Thurston et al., 2002; Waterhouse et al., 2006), and while an ejaculate may surpass the cut-off value for post-thaw motility, this does not guarantee high fertilizing ability (Cremades et al., 2005).

The objectives of this experiment were to test the effect of FTS motility classified as good (>40% motility), moderate (26–39% motility), or poor (<25% motility) from individual sires on pregnancy rate, litter size, and fetal paternity when used in either a first or second insemination in relation to the time of ovulation.

6.3 Materials and Methods

The use of animals for this experiment was approved by the institutional animal care and use committee of the University of Illinois.

6.3.1 Animals and Synchronization of Estrus

This experiment was performed in seven replicates from January to October 2012 at the University of Illinois Swine Research Center. Terminal line gilts (*n* = 290; Genetiporc USA, Alexandria, MN) were moved from a finishing barn into pens in a gestation building between

147 to 180 d of age. Gilts were observed for estrous expression using the back-pressure test while providing fence-line exposure to a mature boar. Gilts that had exhibited estrus (*n* = 268) were then moved into gestation stalls and synchronized by feeding 15 mg/(gilt·d-1) of MATRIX (Altrenogest 2.2mg/mL, Merck Animal Health, Summit, NJ) for 14 d as a top-dress on a standard sow gestation diet. In the first two replicates, gilts were treated with 5.0-mL of PG600 (400IU eCG and 200IU hCG, Merck Animal Health, Summit, NJ) 24-h following the last MATRIX feeding (LMF) to improve synchrony and expression of estrus. However, it was not used in the remaining replicates due to variation in follicle size and limited improvement in expression of estrus. Beginning on the third day following LMF, estrous detection was performed twice daily at 12-h intervals (0700 and 1900h).

6.3.2 Experimental Design

To test the effect of FTS motility in a multiple AI system in relation to time of ovulation, ejaculates were collected from multiple boars and frozen in 0.5 mL straws. Upon thawing, samples were analyzed and classified based on motility as Good (G, ≥ 40%), Moderate (M, 26 to 39%), or Poor (P, 16 to 25%) similar to Pelaez et al. (2006) and Hernandez et al. (2007). Gilts that expressed estrus (*n* = 207) were assigned to receive a total of 4.0 billion sperm in each 1st and 2nd AI performed 24 and 36-h after onset of estrus using the following treatments: 1) P and M (P-M); 2) M and P (M-P); 3) G and M (G-M); and 4) M and G (M-G). Different sires were used in 1st and 2nd inseminations for identification of fetal paternity with sires represented across treatments and inseminations within each replicate (Table 6.1). Gilts assigned to treatment averaged 229 ± 1 d (mean ± SEM) at 1st insemination. Fixed-time inseminations occurred at 24

and 36 h following onset of estrus. Trans-rectal real-time ultrasound (Aloka 500V, Tokyo, Japan) began 12 h following onset of estrus and continued at 12 h intervals to observe the number and size of ovulatory follicles (≥ 6.5 mm) and to determine when ovulation was complete (Knox and Althouse, 1999; Knox et al., 2002) to calculate the insemination to ovulation interval (IOI).

6.3.3 Semen Collection, Freezing, and Evaluation

Boars (*n* = 38, Genetiporc USA, Alexandria, MN) from a commercial genetic supplier used in a regular collection rotation were selected for this experiment. Ejaculates (*n* = 54) were frozen during July 2010 to August 2012 for each boar. Semen was collected and diluted with Modena extender (Swine Genetics International, Cambridge, IA) at a ratio of 1:1 and cooled to 17 °C within 0.5 h after collection and shipped overnight to either the USDA-ARS National Center for Genetic Resources Preservation (Fort Collins, CO) and processed as described by Spencer et al. (2010) or to the University of Illinois (Urbana-Champaign, IL) and processed using the procedures of Ringwelski et al. (2013).

Briefly, upon semen arrival, the sample was evaluated for concentration and motility and centrifuged at 800 x *g* and the supernatant was aspirated. The sperm pellet was re-suspended with AndrohepCryoGuard Cooling Extender (Minitube of America, Verona, WI) to a concentration of 2.8 x 10^9 sperm/mL and held at 5 °C for 2.5 h before dilution to a final concentration of 1.4 x 10^9 sperm/mL withAndrohep CryoGuard Freezing Extender (Minitube of America, Verona, WI). Straws (0.5 mL) were filled and then placed into the Ice Cube controlled rate freezer (Minitube of America, Verona, WI) using the freezing process described by Spencer et al. (2010) and Ringwelski et al. (2013) and stored in liquid nitrogen.

All samples were evaluated three times using the subjective method and later verified using CASA (Hamilton Thorne, Beverly, MA) for post-thaw motility, membrane integrity, and acrosome status at the University of Illinois and then classified based on motility (Table 6.2). Straws were thawed at 50 °C for 20 s, and the contents of the straws expelled into pre-warmed glass tubes at 37 °C. Samples were diluted in Androhep CryoGuard Thawing Extender (Minitube of America, Verona, WI) at 1:40 for motility and 1:400 for determining concentration. Evaluation was performed at 5, 30, and 60 min after thawing. Samples were examined under a phase contrast microscope with a 37 °C heated stage at 200X magnification. Ten fields were examined to evaluate 100 sperm/slide and motility was expressed as a percentage of the total number of sperm cells. Motility analysis was confirmed for all samples using CASA (Hamilton Thorne, Beverly, MA). Fluorescent staining was performed in order to determine membrane integrity using propidium iodide (PI, Sigma Aldrich, St. Louis, MO) and acrosome integrity using Fluorescein isothiocyanate lectin from peanut agglutinin (FITC-PNA, Sigma Aldrich, St. Louis, MO). Samples were thawed and diluted 1:50 in 26 °C Beltsville Thawing Solution® (Minitube of America, Verona, WI) and co-incubated for 10-15 minutes with the fluorescent stains for 15 min and fixed with 0.4% paraformaldehyde in PBS solution. A total of 300 sperm was analyzed for fluorescence using a Carl Zeiss AxioCamHRc (Carl Zeiss Microscopy, LLC. Thornwood, NY) at 400X magnification and the percentage of PI negative and FITC-PNA positive sperm calculated from the total number of sperm evaluated.

6.3.4 Semen Thawing and Insemination

Thawing of straws was carried out in a thermally controlled water bath at 50°C for 20 s. The contents of the straws were expelled into 100 mL plastic AI bottles containing 80 mL of

AndrohepCryoGuard Thawing Extender held in a 26°C water bath. Within 15 min of thawing, intra-cervical insemination was performed using polygel-tipped AI catheters (Minitube of America, Verona, WI). All inseminations were subjectively scored for fluid loss during and in the minute following AI catheter removal with a score of 1 showing little or no fluid lost, 2 exhibiting moderate loss, and 3 with excessive fluid lost. The average score for all 1st and 2nd inseminations was 1.6 ± 0.1 and 1.7 ± 0.0, respectively.

6.3.5 Reproductive Tract Processing for Pregnancy and Litter Responses

Gilts were slaughtered at a local abbatoir on d 32 ± 1.0 following assignment to treatment and insemination. The reproductive tracts were collected and assessed for pregnancy status and the number of normal and abnormal fetuses. Fetuses were counted and weighed, and those with abnormal appearance in size or color and that were ≥1 SD below the average weight of the normal fetuses, were classified as abnormal. A liver sample was removed from each individual fetus for DNA genotyping to determine paternity. Ovaries were examined for the number of corpora lutea and the presence of any abnormalities such as cystic (>12 mm) follicles. Embryo survival was determined by dividing the total number of fetuses by the number of corpora lutea present on both ovaries.

6.3.6 DNA Genotyping

To determine the impact of post-thaw semen quality and IOI, parental identification of fetuses was performed using DNA obtained from the semen of all boars, the blood of all gilts

bred, and the liver of all fetuses. Semen samples were digested using Proteinase-K (Amreseco INC, Solon, OH), sodium dodecyl sulfate (SDS), and dithiothreitol (DTT). DNA was isolated using the ZR-96 Quick-gDNA (Zymo Research, Irvine, CA) according to manufacturer instructions. Based on a set of 30 microsatellite markers recommended by The International Society of Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO), a panel of 14 microsatellite markers was chosen. Primers were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). Primers were assigned to polymerase chain reactions (PCR) based on fragment size and fluorescent tag combinations. Fragment analysis genotyping and PCR reactions were conducted in a 10.0 μL reaction volume comprised of 2.5 μL of DNA evaporated to dryness and 10 μL of PCR media composed of 1 μM forward primers tagged with fluorescently-labeled M13 primer (PET®, NED®, VIC®, or 6-FAM® dye, Life Technologies, Carlsbad, CA), as well as one standard 10μM reverse primer, containing 2 μL of 10x Buffer, 1 μL of 4 mM dNTPs (Invitrogen, Carlsbad, CA), 0.1 μL of HotStarTaq Polymerase (Qiagen, Valencia, CA). PCR was conducted with an initial denaturing temperature of 95 °C for 5 min followed by 34 cycles of a three step process of 94 °C for 1 min, 58 °C for 1.5 min, and 72 °C for 1.5 min with a final step of 72 °C for 1 h. Multiplex PCR products were combined and purified using Promega Wizard® SV96 and sequenced as described by Meyers et al. (2010). Alleles were called using GeneMarker® software (SoftGenetics, LLC) and checked manually. Parentage was determined manually using the genotypes from the known female and the two potential sires.

6.4 Statistical Analysis

Data were analyzed using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC). Continuous response measures were analyzed using the PROC MIXED procedures for significance of the main effects using the *F*-test and differences between least squares means identified using the *t* test. Binary response measures were analyzed using PROC GENMOD and significant main effects and differences between least square means identified using the x^2 test. Binary analyses were performed using a binary distribution and a logit-link. All models for the dependent variables included the main effects of treatment (four levels), IOI for 1st and 2nd AI and their interaction, as well 1st AI and 2nd AI sire and replicate. Of the other variables tested, only ovulation rate ($P < 0.05$) was significant for total and normal fetuses. The other variables such as insemination score, interval from LMF to estrus, and duration of estrus were included as class variables or covariates where appropriate and were not significant ($P > 0.10$) and removed from final models. The assumptions of ANOVA for normal distribution of data were evaluated and tested using PROC UNIVARIATE and for homogeneity of variance using Levene's test. Significant differences were identified at $P \leq 0.05$ and trends at $P > 0.05$ and ≤ 0.10.

Gilts assigned to treatment were excluded from analyses for abnormalities that included an EOI > 60 h (n = 12), ovarian cysts at estrus or at slaughter (n = 21), or uterine infection at slaughter (n = 2).

6.5 Results

Although a total of 4.0×10^9 sperm were used in each AI for all treatments, the actual number of motile sperm inseminated was $0.8 \times 10^9 \pm 0.2$ for P, $1.2 \times 10^9 \pm 0.2$ for M, and 1.7 x

10^9 ± 0.1 for G. The interval from last Matrix™ feeding to estrus was 7.2 ± 0.8 d and the duration of estrus averaged 44.1 ± 1.0 h. The EOI averaged 35.3 ± 0.8 h and the ovulation rate averaged 14.8 ± 0.3 corpora lutea.

There was no treatment x IOI interaction for 1st or 2nd AI for any response measure assessed in this study ($P > 0.10$) and therefore only main effects are presented. Sire used in the 1st and 2nd AI was also not significant (P > 0.10) and was not included in the final models.

6.5.1 Pregnancy Rate and Litter Responses

There was no effect ($P > 0.10$) of treatment (Table 6.3) or IOI (Table 6.4) on pregnancy rate, number of normal fetuses or average fetal weight.

6.5.2 Proportion of Litter and Fetuses from 1st and 2nd Inseminations

Treatment influenced the number of fetuses sired from the 1st and 2nd AI (Table 6.3; $P < 0.05$). The number of fetuses sired from the 1st AI was greater than those sired from the 2nd in all treatments except P-M. The frequency distribution of litters with number of fetuses sired within a litter from the 1st AI and 2nd AI is shown in Figures 6.1 and 6.2, respectively. The IOI also affected number of fetuses sired (Table 6.4; $P < 0.05$), with an increase in number sired when the 1st AI occurred at 0 h relative to ovulation compared to -12 and -24 h but not -36 h. There was no effect of treatment (Table 6.3) on the proportion of fetuses sired from the 1st AI,but there was a trend ($P = 0.10$) for the IOI from the 1st AI to impact the proportion (Table 6.4). The IOI for the 2nd AI influenced the proportion of fetuses resulting in smaller proportions

sired when ovulation occurred at +12 or -24 h compared to 0 and -12 h (Table 6.4, $P < 0.05$) with an increase in number sired from the first AI when ovulation occurred at 24 h compared to 36 and 48 h. There was no effect of treatment (Table 6.3) or IOI (Table 6.4) on the proportion of fetuses sired from the 1st AI ($P > 0.10$). However, IOI influenced the proportion of fetuses from the 2nd AI, and fewer fetuses were sired when ovulation occurred at 24 h compared to 36 and 48 h (Table 6.4, $P < 0.05$).

6.6 Discussion

This study was designed to determine whether differences in the post-thaw motility of frozen-thawed boar sperm used in the 1st or 2nd AI would affect pregnancy establishment, litter size and fetal paternity in relation to the time of ovulation. While we were not able to detect differences in pregnancy rate or litter size with FTS differing by ~10% in motility, changes in the number of fetuses sired by each AI was sensitive enough to identify the effects of treatment and estrus to ovulation interval. Regardless of the order, when poor quality sperm was used there was, on average, 30% fewer fetuses sired when compared to use of moderate sperm. Interestingly, the 1st insemination sired ~60% of all litters except when poor motility sperm was used. In addition, use of poor sperm resulted in fewer litters with more than 6 pigs sired by an insemination. No differences in fertility were evident when good and moderate sperm were used in combination. This work has practical implications since the motility of FTS affects *in-vitro* (Gil et al., 2008) and *in-vivo* (Casas et al., 2010) fertility. Of concern is that FTS motility is already reduced (Medrano et al., 2009; Purdy, 2008) with wide variation noted among boars (Hernandez et al., 2007; Hofmo and Grevle, 2000; Juarez et al., 2011; Woelders et al., 1995) and

between ejaculates within a boar (Pelaez et al., 2006). A primary advantage to using FTS is the ability to disseminate superior genetics to the greatest number of females across great distances. However, individual boars have been shown to have high variability in freezing ability (Thurston et al., 2002; Waterhouse et al., 2006), and superior sires are not necessarily considered "good" freezer boars. This creates problems as superior sires may never or only infrequently produce good quality frozen sperm. If this is the case, they may not be used for cryopreservation, or may be used by doubling the number of sperm per AI dose (Cremades et al., 2005; Foxcroft et al., 2008; Spencer et al., 2010). Unfortunately, doubling the number of sperm per AI dose leads to a decrease in the efficiency of the ejaculate (Almlid et al., 1987; Saravia et al., 2005).

Classification for FTS quality based on motility is actually quite variable (Casas et al., 2010; Hernandez et al., 2007; Thurston et al., 2003) with most ejaculates having less than 60% motility. Detecting fertility effects among different levels of low motility FTS is challenging, but it would appear that paternity testing within the litter offers an opportunity to identify the significance of the subtle differences from *in-vitro* motility measures. Previous studies with cooled semen have shown that the proportion of offspring sired can be used as an indicator of fertility differences between boars when using pooled semen or first and second inseminations from different sires (Dziuk, 1970; Flowers, 1997). From these studies it was reported that sperm from one of the males will often fertilize a majority of the eggs (Dziuk, 1996) and that the seminal plasma components of high fertility boars could improve the fertility of lower fertility boars (Flowers, 1997; Foxcroft et al., 2008). This work is supported by a study which compared homospermic and heterospermic inseminations using FTS, which removes seminal plasma during processing, and did not observe any effect on fertility when using good motility sperm (Pursel and Johnson, 1975). Ringwelski et al. (2013) also used heterospermic inseminations with

FTS to evaluate the effect of interval between inseminations and observed that the paternity of the litter was sensitive enough to detect interactions of treatment with the estrus to ovulation interval. While use of pooled semen (heterospermic) is common in commercial pig production to reduce the risk of lowered pig production in groups of females inseminated with the same semen from a sub-fertile boar, this technique is not used at the genetic selection levels (Knox et al., 2008).

While it is not entirely clear how motility differences in FTS translate into different fertility outcomes, it has been reported that addition of caffeine to FTS *in-vitro* results in increased motility, which provides an improved estimate of viable sperm compared to samples tested without caffeine (Pelaez et al., 2006). This observation would tend to support the data from the present study and many other published studies in which the viability for FTS is often higher than the motility. It is also possible that fertility effects from motility may arise from sperm which are classified as good being more stable or resistant to changes in the female reproductive tract following AI when compared to lower quality sperm (Hernandez et al., 2007). Reduced motility of FTS might also be indicative of higher rates or degree of cryoinjury and result in increased backflow and phagocytosis following AI (Roca et al., 2006a). Supporting evidence for this effect may come from the work of Yamaguchi et al. (2009) who added caffeine and $CaCl_2$to FTS and observed an increase of sperm in the reservoirs and improved fertility following AI, and hypothesized an effect through suppression of the uterine immune response. Our results also support the work of others where differences of ~10% in FTS motility with use of moderate to good quality sperm does not alter fertility *in-vitro* (Eriksson, 2000) or *in-vivo* (Pursel and Johnson, 1975). However, Bwanga (1991) did report increased fertilization rates when ~20% differences occurred within the moderate to good range.

Over 90% of the inseminated spermatozoa are eliminated from the female reproductive tract within 2-3 h after insemination due to backflow and phagocytosis, and this loss is highest when the sperm are weakened due to cryoinjury, as is the case with FTS (Roca et al., 2006b). Additionally, damaged sperm are unable to form a functional sperm reservoir, resulting in a greatly decreased fertile lifespan, lowering the number of sperm cells available to fertilize the oocytes, and decreasing the chances for successful fertilization (Abad et al., 2007b). The sperm reservoir has the ability to select for viable, uncapacitated sperm and to protect them from being damaged (Topfer-Petersen et al., 2002), and reduced binding to the oviductal epithelium may be due to changes in boar sperm membranes associated with storage, which means damaged sperm are excluded from the sperm reservoir (Waberski et al., 2006). While not significant in the present study, this could explain the decrease in pregnancy rate seen when poor motility semen was used in the first insemination.

From a practical standpoint, since ejaculate variation is expected for valuable boars, the question of how or if to use these different quality samples arises. This study suggests that mixing AI doses from FTS of different quality could be used to help increase the rate of genetic improvement with limited effects on pregnancy rate and litter size. Our paternity data suggest that poor FTS should not be used in the 1st AI, but use in the 2nd AI with moderate or good can be used to improve fertility. Extrapolating our data for pig production for every 100 sows mated with the use of a fertility index based on the treatment means for pregnancy and number of healthy fetuses suggests a 10 to 13% step-wise reduction in potential pigs produced from the use of M-G (756), G-M (711), M-P (674), and P-M (561). Based on this index, the data indicate that an importance to the order of insemination quality. While it is uncertain what effects use of only poor, moderate or good would have on fertility, data from the present study and others suggest

that increased fertility would not be expected if only good or only moderate sperm were used when compared to a mix of the two qualities.

Single inseminations would be desirable for use of cooled or FTS and could each result in good fertility if they occurred at the optimal time prior to ovulation (Soede and Kemp, 1995; Waberski et al., 1994). However, variation in the estrus to ovulation interval has been reported in mature gilts (Ringwelski et al., 2013; Spencer et al., 2010) and in weaned sows (Nissen et al., 1997; Soede and Kemp, 1995; Weitze et al., 1994). This variation causes some inseminations to occur too early or too late resulting in decreased pregnancy rates and litter sizes with cooled sperm (Almeida et al., 2000; Nissen et al., 1997; Soede and Kemp, 1995) or with FTS (Ringwelski et al., 2013; Spencer et al., 2010). As a result of this variation and the inability to predict ovulation, the industry has adopted a protocol where two inseminations are spaced 18 to 24 h apart for cooled semen which results in improved fertility compared to the use of single inseminations (Flowers and Esbenshade, 1993; Koketsu, 2005; Lamberson and Safranski, 2000). The timing and spacing of inseminations for FTS is even more critical since the duration of sperm fertility *in-vivo* is reduced compared to cooled semen (Waberski et al., 1994), and single inseminations with FTS often lead to reduced fertility (Almlid et al., 1987; Johnson, 1985). Studies that control ovulation time have been successful with a single AI occurring 6 h ahead of ovulation but not following ovulation (Bertani et al., 1997). Spencer et al. (2010) also did not observe an effect of single compared to double insemination using FTS, which may have occurred due to a fixed AI timing relative to detection of estrus and not a practical AI schedule for use. Surprisingly, we did not observe an effect of the estrus to ovulation interval on pregnancy rate or litter size but did detect differences in the paternity from the 1st or 2nd AI. It has been reported that ovulation in gilts usually occurs between 33-42 h after onset of estrus

(Almeida et al., 2000; Bortolozzo et al., 2005), with induced prepubertal gilts showing an average ovulation time of 33 h (Spencer et al., 2010) while mature synchronized gilts ovulated at 37 h (Ringwelski et al., 2013). According to previous studies, the life span of FTS *in-vivo* is approximately 12 h (Waberski et al., 1994; Watson, 2000) but to match the fertility of cooled semen, an AI with FTS must occur within 4 h before ovulation. Based on these data, we fixed inseminations at 24 and 36 h after onset of estrus and observed a 35 h estrus to ovulation interval. Approximately 34% of gilts ovulated by 24 h, 41% by 36 h, 20% by 48 h, and 4% by 60 h. With our fixed time inseminations, the majority of gilts ovulated within 12 h of receiving an insemination. It was interesting to note that the majority of inseminations were all or none while ~1/3 of all litters was from the 1st and 2nd AI.

6.7 Conclusions

The results of this study indicate that when using a double insemination with FTS at 24 and 36 h after onset of estrus in mature gilts, used combinations of semen quality did not result in effects on pregnancy rate or litter size, but use of poor quality sperm reduced fetal paternity. There appears to be no advantage for use of good compared to moderate sperm in fertility. Fetal paternity seems a much more sensitive tool for evaluating the effects of FTS quality and estrus to ovulation effects and interactions than other measures. While effects are uncertain, an index evaluation suggested that pig production could be affected by order of insemination and sperm quality.

6.8 Tables and Figures

Table 6.1 Example allocation of gilts to treatment within a replicate by sire for 1st and 2nd inseminations with frozen thawed boar sperm classified as having Poor (P), Moderate (M), or Good (G) motility. Gilts were assigned in numerical order as they expressed estrus. For each treatment row, three boars were randomly selected within motility class and by their allelic distinction from the other boars used within the row. Also within a row, the same boar with Moderate sperm was represented across all treatments. This treatment design allowed direct comparisons among treatments and indirect comparisons without performing all comparisons. Treatments were designed to obtain the best estimates without performing all comparisons between boars, similar to Collins, et. al (2008).

Treatment			
P-M	M-P	G-M	M-G
$1^{(A,B)}$	$2^{(B,A)}$	$3^{(C,B)}$	$4^{(B,C)}$
$5^{(D,E)}$	$6^{(E,D)}$	$7^{(F,E)}$	$8^{(E,F)}$
$9^{(A,E)}$	$10^{(E,A)}$	$11^{(C,E)}$	$12^{(E,C)}$
$13^{(D,B)}$	$14^{(B,D)}$	$15^{(F,B)}$	$16^{(B,F)}$

Numbers (1 to 16) represent the first 16/36 gilts assigned to treatment in a replicate based on order of estrus expression with superscripts indicating some of the 38 sires that could be used (A-F) in order of insemination.

Table 6.2. Means (± SEM) for frozen-thawed boar sperm measures of motility, viability, and live sperm with intact acrosomes from ejaculates used in treatment classification based on motility.

		Post-thaw sperm measures		
Motility class[a]	n[b]	Motility, %	Viability, %	Live sperm with intact acrosomes, %
Good	16	43.5 ± 0.8[x]	51.9 ± 2.3[x]	80.3 ± 1.5
Moderate	23	31.3 ± 0.9[y]	40.5 ± 2.2[y]	77.9 ± 1.5
Poor	15	20.2 ± 1.1[z]	28.2 ± 2.0[z]	74.8 ± 1.8

[a]Evaluation for motility was performed 5 min after thawing using microscopic assessment and then confirmed using CASA for all ejaculates and classified motility as Good (G, ≥ 40%), Moderate (M, 26 to 39%), or Poor (P, 16 to 25%).
[b]n = number of ejaculates collected from 38 individual boars.
[x,y,z] Within a column, means without a common superscript are different ($P < 0.05$).

Table 6.3. Means (± SEM) for pregnancy rate, number of fetuses, embryo survival, and fetal paternity from each insemination as affected by 1st and 2nd inseminations with frozen thawed boar sperm classified as having Poor (P), Moderate (M), or Good (G) motility.

Treatment[a]	n[b]	Pregnant, %[c]	Total fetuses/ litter	Normal fetuses/ litter	Embryo survival, %[d]	Proportion of litter from 1st AI, %	Proportion of litter from 2nd AI, %	Total number of fetuses from 1st AI*	Total number of fetuses from 2nd AI*
P-M	41	57.1 ± 8.0	9.2 ± 1.1	8.7 ± 1.0	61.5 ± 6.5	38.1 ±9.4	61.4 ± 9.2	3.1± 0.9^{x}	5.7± 1.1^{x}
M-P	43	67.4 ± 7.2	9.1 ± 0.7	8.8 ± 0.7	63.1 ± 4.4	67.6 ± 8.6	31.7 ± 8.5	7.2± 1.1^{y}	2.6± 0.8^{y}
G-M	45	71.1 ± 6.8	9.5 ± 0.7	9.3 ± 0.7	61.6 ± 4.6	65.5 ± 7.0	33.6 ± 7.0	6.4± 0.9^{y}	3.0± 0.8^{y}
M-G	42	75.6 ± 6.8	10.0 ± 0.8	9.6 ± 0.7	65.5 ± 4.4	65.0 ± 7.5	34.8 ± 7.6	6.3± 1.0^{y}	3.6± 0.9^{y}

[a]Gilts received a total number of 4.0 billion FTS assessed as P (20.2 ± 1.1%) , M (31.3 ± 0.9%), or G (43.5 ± 0.8%) in 80 mL of extender at 24 and 36 h after detection of estrus. The number of motile FTS in each AI was 0.8 x 10^9 ± 0.2 for P, 1.2 x 10^9 ± 0.2 for M, and 1.7 x 10^9 ± 0.1 for G.

[b]Of all animals assigned to treatment, gilts were excluded from analyses due to an abnormally long estrus to ovulation interval (> 60 h, n = 12) or if ovarian and reproductive tract abnormalities such as ovarian cysts or uterine infection were evident at estrus or slaughter (n = 23).

[c]Determined at slaughter at 31 to 35 d following AI.

[d]Embryo survival determined from number of normal fetuses/ number of corpora lutea.

*Within a column, means without a common superscript are different (P < 0.05)

Table 6.4. Means (± SEM) for pregnancy rate, number of fetuses, embryo survival, and fetal paternity from each insemination as affected by interval from insemination to ovulation when using frozen thawed boar sperm.

Insemination to Ovulation (h)[a]		n[b]	Pregnant, %[c]	Total fetuses/ litter	Normal fetuses/ litter	Embryo survival, %	Proportion of litter from 1st AI, %†	Proportion of litter from 2nd AI, %*	Total number of fetuses from 1st AI*	Total number of fetuses from 2nd AI*
AI 1	AI 2									
-36	-24	7	57.1 ± 20.2	6.3 ± 1.3	6.3 ± 1.3	44.7 ± 13.1	70.0 ±23.8^{xy}	30.0 ± 23.8^{xy}	4.8 ± 2.1^{xy}	1.5 ± 1.2^{x}
-24	-12	32	62.5 ± 8.7	8.4 ± 0.8	8.1 ± 0.9	57.8 ± 5.4	40.9 ± 9.8^{x}	57.7 ± 9.7^{y}	3.4 ± 0.9^{x}	5.4 ± 1.1^{y}
-12	0	66	70.0 ± 5.7	9.8 ± 0.7	9.6 ± 0.6	65.1 ± 4.3	49.4 ± 6.8^{x}	51.3 ± 6.7^{y}	4.9 ± 0.8^{x}	5.0 ± 0.8^{y}
0	12	55	68.5 ± 6.3	10.2 ± 0.1	9.8 ± 0.7	66.5 ± 4.1	80.1 ± 5.5^{y}	18.3 ± 5.4^{x}	8.4 ± 0.9^{y}	1.6 ± 0.6^{x}

[a]Estrus detection and ultrasound performed at 12 h intervals (0600 and 1800 h) and gilts received 4.0 billion total sperm assessed as having Poor (P, 20.2 ± 3.4%) , Moderate (M, 31.3± 4.2%), or Good (G, 43.5± 3.2%) motility in the 1st or 2nd insemination at 24 and 36 h after detection of estrus.

[b]Gilts (n = 12) could not be included due to inability to perform repeated trans-rectal ultrasound to confirm ovulation time.

[c]Determined at slaughter at d 31 to 35 following AI.

*Within a column, means without a common superscript are different ($P < 0.05$).

†Within a column, means without a common superscript are different ($P = 0.1$).

Figure 6.1. The effects of 1st and 2nd inseminations using combinations of frozen-thawed sperm (FTS) classified by post-thaw motility as Poor (P) Moderate (M) or Good (G) from unique sires on the frequency of litters with defined numbers of fetuses sired by the 1st AI within a litter.

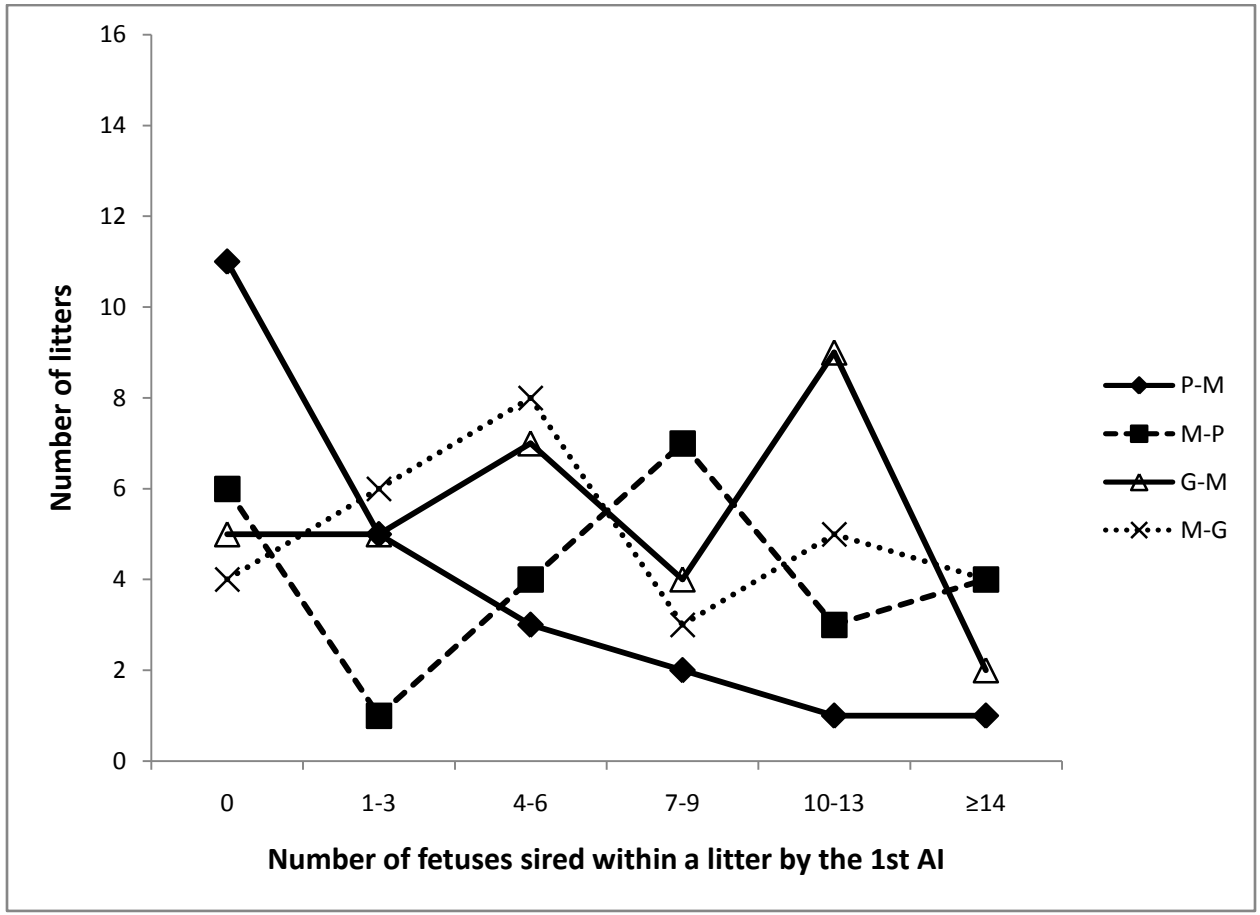
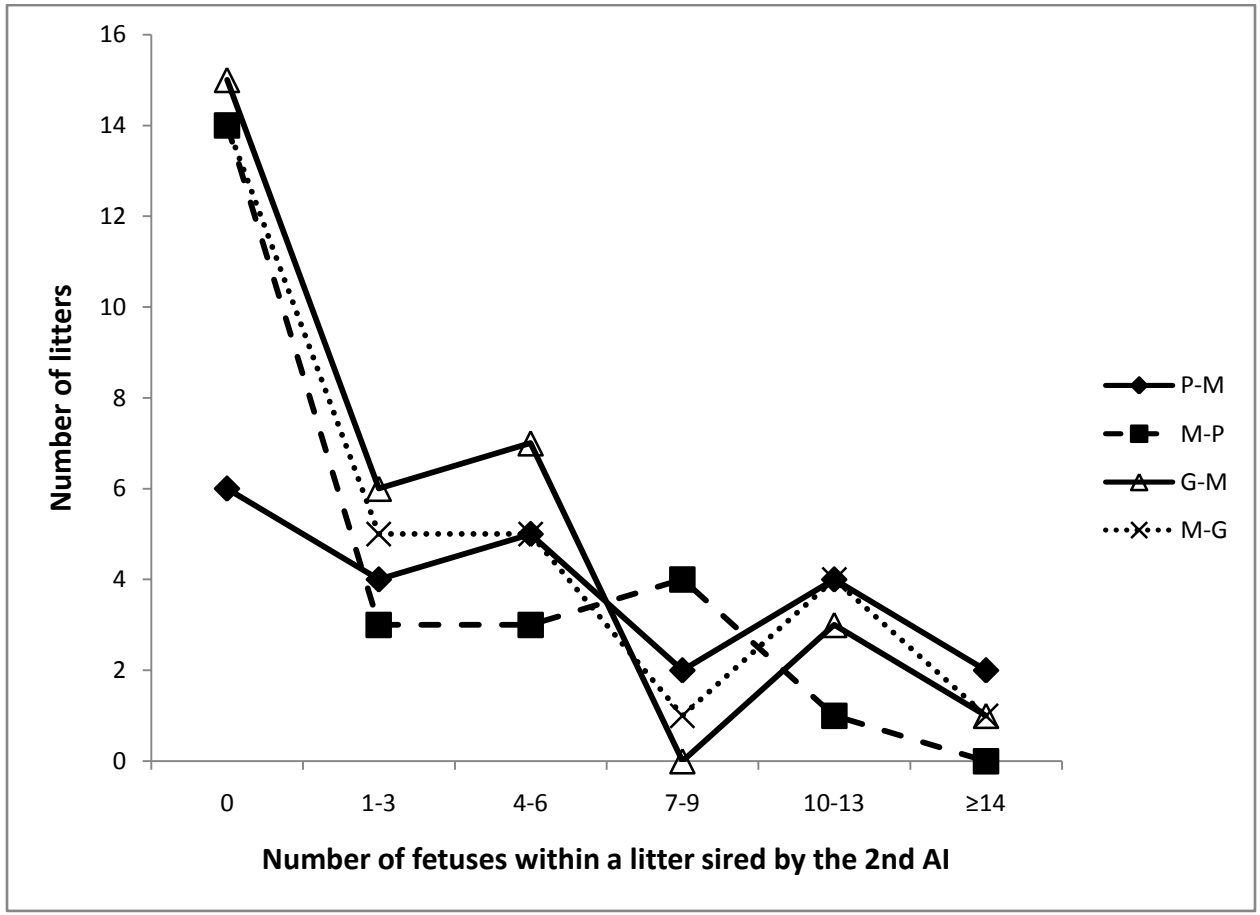


Figure 6.2. The effects of 1st and 2nd inseminations using combinations of frozen-thawed sperm (FTS) classified by post-thaw motility as Poor (P) Moderate (M) or Good (G) from unique sires on the frequency of litters with defined numbers of fetuses sired by the 2nd AI within a litter.



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Appendix A: Boar Sperm Cryopreservation Protocol

Semen Processing and Freezing:

1) Upon arrival in the lab, Process semen by first evaluating the volume. Divide the volume equally between the correct numbers of 250 mL centrifuge tubes. Do not exceed 200 mL per tube. Place centrifuge tubes in 17^0 C water baths in Coolatron®.

2) Prepare a 2mL subsample in order to evaluate concentration and motility.
   - Concentration: *Evaluate at a 1:100 Dilution*. Load both sides of a Hemocytometer and count all sets of 5 diagonal boxes. Calculate Total Number of Sperm cells in Ejaculate (TSE).
     - [1:10] 100 µL of Sperm with 900µL of *Androhep CryoGuard Extender*
     - [1:100] 100 µL of 1:10 dilution and mix with 880 µL of *Androhep CryoGuard Extender* and 20 µL Formaldehyde
     - ***Calculations***: $(1)(AveHemoCount)(5)(10^2)(10^4)$= Concentration

   - Motility: *Evaluate at a 1:40 Dilution*. Allow the sample to warm in a 37°C warming block for 20 minutes prior to evaluation. Count total number of motile and progressively motile sperm. Evaluate 10 fields and calculate average motility.
     - [1:4] 100 µL Sperm with 300 µL of *Androhep CryoGuard Extender*
     - [1:40] 100 µL of 1:4 dilution and mix with 900 µL of *Androhep CryoGuard Extender*

3) Allow samples to sit in 17^0 C to equilibrate temperature and pH. Weigh centrifuge tubes to determine volume of sample. (Volume is used to determine TSE) ***Total Time in 17^0 C: 1-1 ½ Hours***

4) Centrifuge at 16-17^0 C at 800x G (~1700 rpms) for 12.5 minutes in the 250 mL centrifuge tube

5) Aspirate the supernatant out of the centrifuge tubes leaving the sperm pellet, using the sterile hood with the vacuum and flask.

6) Weigh sperm pellet. Based on the calculated final volume, re-suspend the sperm pellet using the calculated volume of cooling extender (*Androhep Cooling Extender*®) and mix using a pipette. Cooling extender and pellet weight should account for 50% of the final volume. *The calculated final volume should result in 1.4 x 10^9 Sperm/mL.
   - *Concentration x Volume = TSE*
   - *TSE / (1.4 x 10^9 Sperm/mL) = Freezable Volume*

7) Place samples and freezing extender/cryoprotectant (*Androhep Freezing Extender*®) in 5^0 C water baths in cold room. Allow to cool slowly over 2½ hours.

8) Add calculated volume of freezing extender and mix gently using a pipette. * Should equal total calculated freezing volume.

Semen Packaging

1) Label the appropriate number of 0.5 mL plastic straws (*Total freezable volume/2*) and place them in the cold room to allow equilibration of temperature to 5 °C.

2) Immediately fill and seal labeled 0.5 mL plastic straws in cold room. Shake the straw magazine to move the air bubble to the middle of the straw. This will prevent straws from bursting upon thawing.

3) Load the freezing program. Allow Ice Cube to reach start temperature of 2 ^{0}C. Open the lid and place straw racks in controlled freezer. Allow the machine to equilibrate at 2 ^{0}C. Start the program.

Freezing Rate Table

Time	Temperature	Slope (Degree/Minutes)
0	2^0 C	0
3 mins	-4^0 C	-2^0 C/ min
.87 mins	-30^0 C	$+30^0$ C/min
1 mins	-25^0 C	-5^0 C/min
11 mins	-140^0 C	-10^0 C/min

4) After the cycle has finished, stop the machine and remove straw racks.

5) Plunge straws into LN2 and sort straws into 8 straws per goblet and 2 goblets per cane.

6) Transfer all of the canes to the appropriate liquid nitrogen tank.

Appendix B: Boar Sperm Evaluation Protocol

Motility Evaluation

1. Thaw a straw of semen in a water bath at 50 °C for 20 seconds. Plunge the contents of the straw into a pre-warmed glass test tube in a 37 °C heating block.
2. *Evaluate at a 1:40 Dilution*. Allow to warm in warming block for 5 minutes prior to evaluation. Count the total number of motile and progressively motile sperm. Evaluate 10 fields and calculate average motility.
    - [1:4] 100 µL Sperm with 300 µL of *Androhep CryoGuard Extender*
    - [1:40] 100 µL of 1:4 dilution and mix with 900 µL of *Androhep CryoGuard Extender*

3. Incubate in thaw extender at 37 ºC for 5 minutes.
4. Place sample on pre-warmed slide and place a cover-slip over the sample.
5. Allow slide to sit on the warmed stage for 1 to 2.5 minutes.
6. Using a phase-contrast microscope with a 20x power objective, count 10 fields of 10 cells each, recording motile sperm.
7. Repeat the motility evaluation at 30 and 60 minutes post-thaw in order to determine longevity of motile sperm.

Fluorescent Staining

1. Thaw a straw of semen in water bath at 50 °C for 20 seconds. Plunge contents of straw into a glass test tube in a 37 °C heating block.
2. Prepare staining media in glass test tubes at room temperature (26 °C)
    - 500 µL BTS Extender
    - 7 µL Propidium Iodide (PI)
    - 15 µL Fluorescent Lectin with Peanut Allugitinin (FITC-PNA)

3. Pipette 10 µL of stock semen sample into staining media. Return stock sample to 37 °C warming block until 30 and 60 minute evaluations.
4. Allow samples to co-incubate with staining media for 10-20 minutes at room temperature (26 °C).
5. Fix samples by adding 2 µL of 0.4% Paraformaldehyde solution.
6. Pipette 20 µL of the fixed sample onto a microscope slide and cover with cover slip.
7. Allow slide to sit for 10 minutes to allow sperm cells to settle.
8. Using the Zeiss Axioskop®, evaluate the sample by counting 200 total sperm in multiple fields to determine:
    - Percent Viable: Total Non- FITC or Sperm – Bright Filter
    - Percent Live Acrosome Reacted: Non- stained sperm with green acrosome –FITC Filter
    - Percent Dead/Membrane Compromised: stained (red) sperm – PI Filter

9. Compare sperm cells imaged for PI and FITC stains to Bright Field images to determine Percent Viable, Percent Live with Acrosome Reaction, and Percent Dead/Membrane Compromised.

10. Repeat steps 3-9 at 30 and 60 minutes post thaw.

Appendix C: Fetal Liver Digestion, DNA Isolation, and PCR Protocols for Genotype Determination

Tissue Digest – Used for boar semen samples

1. Aliquot 75 µL of tissue samples.
2. Add 120 µL of DNA Extraction Buffer, 15 µL Proteinase-K, 24 µLsodium dodecyl sulfate (SDS), and 10 µLdithiothreitol (DTT).
3. Incubate at 60 ºC for 12 hours.

DNA Isolation

1. Add 75 µ of tissue digest to 300 µL of Genomic Lysis Buffer ™ in 96 well plate. Vortex well.
2. Transfer 300 µL of mixture to Silicon-A™ Plate and Collection plate. Centrifuge at 2,500 x *g* for 5 min.
3. Add 200 µL of Pre-Wash Buffer to each well. Centrifuge at 2,500 x *g* for 5 min.
4. Add 300 µL of g-DNA Wash Buffer to each well. Centrifuge at 2,500 x *g* for 5 min.
5. Transfer Silicon-A™ Plate onto an Elution Pate. Add 50 µL of DNA Elution Buffer to each well. Incubate for 5-10 minutes at room temperature. Centrifuge at 2,500 x *g* for 5 min.
6. Eluted DNA store at – 20 ºC until future use.

PCR Protocol

1. Create PCR master mix using the following tables.

4 Marker PCR	**1X**	**100X**
DNA (Per Well)	3	3
10X PCR Buffer	1	100
4mM dNTPs	0.5	50
P1	0.125	12.5
P2	0.25	25
P3	0.125	12.5
P4	0.25	25
P5	0.125	12.5
P6	0.25	25
P7	0.125	12.5
P8	0.25	25
M13	.25	25
Taq	0.07	7
Optima H20	3.68	368
TOTAL	**10**	**1000**

3 Marker PCR	**1X**	**100X**
DNA	3	3
10X PCR Buffer	1	100
4mM dNTPs	0.5	50
P1	0.125	12.5
P2	0.25	25
P3	0.125	12.5
P4	0.25	25
P5	0.125	12.5
P6	0.25	25
M13	.25	25
Taq	0.07	7
Optima H20	4.15	415.5
TOTAL	**10**	**1000**

Primer and M13 Tag Sets				
	Blue	**Green**	**Yellow**	**Red**
M 13 Tag	FAM®	VIC®	PET®	NED®
Primer 1	SW 240	SW24	SW122	SO178
Primer 2	SO155	SO228	SW2406	SW632
Primer 3	SO226	SW857	SO002	SO101
Primer 4	SO090	SW830		

2. Run PCR Program as follows

1. 95° C for 5:00 minutes
2. 94° C for 1:00 minutes
3. 58° C for 1:30 minutes
4. 72° C for 1:30 minutes
5. 34X to Step 2
6. 72° C for 60:00 minutes
7. 10° C for 5:00 minutes
8. END

Post-PCR Processing:

1. Combine 3 µL of Blue, Green, and Yellow PCR Product with 5 µL of Red PCR Product.
2. Elute with 75 µL Optima H_2O.
3. Aliquot 4 µL of eluted PCR product into Promega WizardR SV96 ®binding plates.
4. Submit to Keck Center for Sequencing.

Appendix D: Preliminary Thaw Test Data

Thawing of straws was carried out in a thermally controlled water bath at 37 °C for 10 and 20s. A total of 24 straws were thawed, (n = 2 straws per ejaculate). Samples were diluted in Androhep CryoGuard Thawing Extender (Minitube of America, Verona, WI) at 1:40 for motility and held in a 37 °C heating block. Evaluation was performed at 5 and 60 min after thawing. Samples were examined under a phase contrast microscope with a 37 °C heated stage at 200X magnification. Ten fields were examined to evaluate 100 sperm/slide and motility was expressed as a percentage of the total number of sperm cells. Fluorescent staining was performed in order to determine membrane viability using propidium iodide (PI, Sigma Aldrich, St. Louis, MO). Samples were thawed and diluted 1:50 in 26 °C Beltsville Thawing Solution® (Minitube of America, Verona, WI) and co-incubated for 10-15 min with the fluorescent stain for 15 min and fixed with 0.4% paraformaldehyde in PBS solution. A total of 300 sperm was analyzed for fluorescence using a Carl Zeiss AxioCamHRc (Carl Zeiss Microscopy, LLC. Thornwood, NY) at 400X magnification and the percentage of PI negative sperm was calculated from the total number of sperm evaluated.