

THESIS

APPLYING IN VITRO-PRODUCED EMBRYOS AND SEXED SPERM TO DAIRY
CATTLE REPRODUCTION.

Submitted by

Sara-Lesley Rasmussen

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2011

Master's Committee:

Advisor George E. Seidel Jr.

James Graham

Patrick McCue

ABSTRACT OF THESIS

APPLYING IN VITRO-PRODUCED EMBRYOS AND SEXED SPERM TO DAIRY CATTLE REPRODUCTION.

This study compared the pregnancy rates between embryo transfer of bovine embryos produced in vitro with sexed vs control sperm and artificial insemination (AI) using sexed and unsexed sperm. Cleavage rates for oocytes fertilized with sexed vs control sperm were not different for two of the three bulls used, but were lower ($p < 0.05$) for the third bull sexed (44%) vs control sperm (70%). There were fewer transferable blastocysts produced per oocyte with sexed sperm (9-19%) than for unsexed sperm (18-26%); ($p < 0.05$). All cows were on an Ovsynch program to synchronize ovulation. Respective 60 d pregnancy rates at two Colorado dairies were as follows: control AI (43%, n=88; 43%, n=44); AI with X-sorted sperm (34%, n=82; 34%, n=62); embryo transfer (ET) with in vitro-produced (IVP) embryos using unsexed sperm (22%, n=68; 21%, n=39); and ET with IVP embryos using sexed sperm (7%, n=72; 37%, n=40). The pregnancy rate (day 60) for AI using sexed sperm was 78% of that of control sperm. ET pregnancy rates were generally lower than AI rates. At one dairy, abortions between days 32 and term were higher for X-sort ET pregnancies (79% n=14) than for AI control pregnancies (20% n=40); ($P < 0.001$). However, the other dairy experienced only a 12%, (n=17) abortion rate for transferred embryos produced from X-sorted sperm. The sex ratio of calves was similar to previous studies for AI with control sperm (52% bull calves, n=50), AI with

X-sorted sperm (12% bull calves, n=40); ET with IVP embryos using unsexed sperm (50% bull calves, n=18); and ET with IVP embryos using sexed sperm (11% bull calves, n=18). Findings from this experiment indicate that embryo production with sexed sperm is not successful enough to be applied to large-scale dairies that already have successful breeding programs in place.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the USDA. This research was supported by NRI Grant no. 2006-55203-17390 from USDA-CREES. Select Sires, Inc. and XY, Inc. donated semen, and Bionich Life Sciences donated materials.

Thank you to Dr. McCue and Dr. Graham who agreed to be members of my committee and took the time to review my thesis project.

I was privileged and greatly humbled to have been able to spend a year in Dr. Seidel's lab prior to entering the Professional Veterinary Medicine Program at CSU. He teaches students about more than just reproduction and meticulous analysis of research quality but shares his life. He and his wife Sarah allow people to experience their ranch life.

A special thanks to Zell Brink. She taught me the art of careful planning and patience for experimentation and was good company.

Thanks to Dr. Kevin McSweeney who introduced me to the dairy industry. Kevin's generosity with his practice allowed me the opportunity to gain practical experience working with food animals. Thanks to his co-workers Dr. Jay Hudson who inseminated and transferred embryos, Aaron Shiflett who inseminated and relayed data, and technicians Alex, Nick, Ashley, Andres, and Toni.

Thank you to John Johnson and crew and Jim and Judy Docheff and sons, Jay, Justin, and Joe, for allowing us the use of their cows.

Dr. Jennifer Barfield who was a helpful mentor. Thank you to Veterinary Students Michelle Turk and Ashley Carr who helped with field work and provided moral support.

Thank you Moises Barcelo-Fimbres, Lincoln Amorim, Fernanda Morales Pliego, Josh Walker, Scott Purcell, Melissa Hudson, Beth Spizziri, and Mindy Meyers for training and help in each of your specialties.

Thanks goes to my husband, Trent, daughter, Star and son, Talon who have endured my college career. Their encouragement has helped me realize my lifelong goals.

Thank you to my Dad, Mom, Aunt Marilyn and Cousin Jose who have supported my interests throughout my life. Finally, thank you to Amber Kelley my children's second mom.

TABLE OF CONTENTS

CHAPTER I. INTRODUCTION.....	1
Chapter II. LITERATURE REVIEW.....	5
1. Fertility in Dairy Cattle.....	5
2. Assisted Reproduction.....	8
<u>Sexing Sperm</u>	8
<u>Artificial Insemination</u>	11
<u>Embryo Transfer</u>	12
3. In Vitro Production of Bovine Embryos.....	15
<u>Quality Control of IVP Bovine Embryos</u>	15
<u>Oocytes</u>	18
<u>Sperm</u>	20
4. Blastocyst Production and Relative Stages of Development.....	21
5. Success Rates with in Vitro-Produced Embryo Transfer and Calving in Cattle.....	22
Chapter III RESEARCH PROJECT.....	24
Introduction.....	24
Materials and Methods.....	25
<u>In Vitro Fertilization and Culture Media</u>	25

<u>Embryo Production</u>	26
<u>Cryopreservation of Embryos</u>	29
<u>Animals</u>	30
<u>Experimental Design</u>	32
<u>Statistical Analysis</u>	32
Results.....	32
<u>IVP Embryos</u>	32
<u>Transfers and Inseminations</u>	34
<u>Pregnancy Rates by Treatments</u>	36
<u>Abortion and Calving Data</u>	38
Discussion.....	41
<u>IVP Embryos</u>	41
<u>Technician and Dairy Differences</u>	43
<u>Preganancy Rates</u>	43
<u>Abortion and Calving Data</u>	44
Conclusions.....	45
Appendix 1 Cryopreserved, In Vitro-Produced Embryos.....	47
Work Cited.....	48

I INTRODUCTION

Low pregnancy rates of dairy cattle affect producers economically, therefore modifying reproduction programs to improve pregnancy rates and genetic quality would be valuable. Having multiple options to manage herd fertility allows farmers to choose a program for the needs of their particular operation. Development and refinement of a system to sort sperm by DNA content on the basis that bovine X sperm contain about 4% more DNA than Y sperm has led to wide use of this technology for cattle breeding (Schenk and Seidel, 2007; Garner and Seidel, 2008; De Jarnette et al., 2010). Likewise, embryo technologies have potential to improve genetics and fertility in certain situations (Al-Katanani et al., 2002; Hansen and Block, 2004; Hansen, 2006). Due to exceedingly low fertility of dairy cows, changes are needed in reproductive management, and could include incorporation of available reproduction technologies.

In the dairy industry many factors need to be considered for an overall successful reproduction program, including herd health, specific management practices, and costs. Options, such as the use of sex-sorted sperm in a successful Artificial insemination (AI) program, increase production of on farm replacement heifers, and decrease the need for outside replacement heifers. Purchase of replacement heifers can be expensive and may pose a biosecurity hazard as infectious diseases can be brought to a premises. Increasing the number of in-herd replacement heifers could increase the rate of genetic gain on farms and justify investing more on quality semen (DeVries et al., 2008). The value of

female dairy calves generally is 2-3 times that of males (Wheeler et al., 2006), and the freemartin condition would not occur if twin female pregnancies occurred. In addition, AI using unsorted or Y-sorted beef sperm could be used to produce more valuable calves from less genetically valuable cows, if cows with better genetics are bred with sexed sperm for replacement heifers.

Another potential tool to improve reproduction on dairies is the use of embryo transfer (ET). Twinning occurs in almost 7% of pregnancies in high producing dairy cattle, and decreased twinning is a benefit of ET. A twin pregnancy often results in mortality of one or both calves, and health problems for twin calves born alive and for the dam (Silva-del-Rio et al., 2007, 2009; Hossein-Zadeh et al., 2009). Twins also tend to result in less milk production following parturition (Lucy, 2001). Pregnancy rates can be improved using embryo transfer in some cows with intrinsic fertility problems or heat stress (Drost et al., 1999; Al-Katanani et al., 2002; Hansen and Block, 2004).

This project was designed to provide objective information about the success and benefits of using sexed versus control sperm for AI and for transfer of embryos produced by in vitro-fertilization (IVF). Commercial farms, with their diverse conditions and protocols, yield results for ET that tend to vary by farm (Stroud and Hasler, 2006), making it important to sample many conditions to get an idea of the potential for particular technologies.

Historically, the production of embryos in vitro (Hasler, 2003; Lonergan, 2007) and the logistics of cryopreservation and transfer of IVP bovine embryos have not been very efficacious (Al-Katanani et al., 2002; Hansen, 2006). A protocol that results in a high yield of viable embryos that are easy to thaw and transfer on farm would be useful.

However, IVP embryos can result in post partum abnormalities such as abnormal offspring syndrome (Lonergan, 2007; Lonergan and Fair, 2008; Hansen and Block, 2004). New methods for producing embryos that result in embryos that are similar to those produced in vivo need to be developed. This could improve both the viability of cryopreserved embryos and the normality of resulting offspring (Al-Katanani et al., 2002; Rizos, 2003; Hansen, 2006).

There is limited information on pregnancy rates from embryos produced by in vitro fertilization using sexed sperm (Xu et al., 2006). Although sexing sperm achieves 85-95% accuracy of the selected sex (Garner and Seidel, 2003; Garner, 2006), it is detrimental to sperm and results in fewer IVP embryos than unsorted sperm (Hansen, 2006). However, an investigation demonstrated comparable gene expression of certain developmental genes by early embryos produced with sorted and unsorted sperm (Bermejo-Alvarez et al., 2010). The oocyte probably corrects some spermatozoal chromosomal damage, if present, so the embryo can develop normally to the blastocyst stage; the embryo may be affected by retained defects (Fernandez-Gonzalez et al., 2008). Therefore, producing viable offspring starting with embryos produced in vitro with sexed sperm needs further evaluation. Supportive data on incorporating sexed sperm and embryo transfer as well as other assisted reproduction technologies (ART) tools into working farms may facilitate use of these technologies and decrease costs.

Successful reproduction programs are often facilitated by an effective estrus synchronization program that leads to precisely timed submission of cattle for AI without compromising fertility, with decreased requirements for estrus detection (Lucy, 2007). This is particularly helpful in conditions like heat stress, where signs of estrus are often

less obvious or undetectable (Al Katanani, 2002). Synchronization programs may require modifications to optimize use of sexed sperm, a complication if other cows are on a different insemination schedule (DeJarnette, 2010).

Other factors affecting fertility, that can be considered ancillary variables, include individual dairy management and body condition score (BCS). Although these factors were not evaluated in this study, they may be quite important in some situations (Stroud and Hasler, 2006; Seidel and Schenk, 2008). Fertility, productivity, and health of cows are also aspects that need to be balanced for dairy herds to be profitable. Protocols that maximize embryo health and viability can be used to improve reproduction. For systems that incorporate in vitro-produced embryos, items to be optimized include culture media and preparation, incubation conditions, embryo handling, methods of cryopreservation, embryo transfer, and hormone treatment of recipients (Hansen and Block, 2004; Hansen, 2006).

This thesis research was part of a larger field trial at dairy farms in Colorado (and Florida) that also will include economic analyses. This thesis covers findings through calving and determining sex of offspring on the two Colorado dairies only. The main endpoint will be pregnancy rates with other endpoints such as embryonic death. The resulting information about ART should be valuable for researchers and producers.

II LITERATURE REVIEW

1. Fertility in Dairy Cattle

Due to the ever-increasing demands on dairy cattle for increased milk production and the unfortunate negative correlation between milk production and fertility, dairy cattle fertility has decreased (Lucy, 2001, 2003, 2007). Between 1970 and 1996 there was a drop in pregnancy rates of 25 percentage points while milk production increased markedly (Lucy, 2001; Hasler, 2006). This likely can be attributed to partitioning of nutrients from adipose tissue to milk and other energy requiring processes, leading to decreased body condition and sacrificed reproductive function. This negative energy balance is linked to four main categories of infertility as defined by Lucy (2007).

The first category, anovulatory or behavioral anestrus, is a normal postpartum condition, but is defined as a problem when it persists beyond the voluntary waiting period (defined as the number of days postpartum when breeding is initiated, usually around 50-60 days in milk). Cows that have higher milk production also have a longer period between parturition and the next ovulation, but this in itself may not be directly correlated with fertility (Lucy, 2007). Hypothesized mechanisms for the increased period between parturition and ovulation include decreased concentrations of insulin and IGF-1 due to negative energy balance. This in turn causes a decrease of LH secretion, regulated at the hypothalamus and pituitary. This is thought to cause disruption of normal

folliculogenesis. In lactating cows the dominant follicle becomes larger than that of heifers, while the blood concentration of estradiol-17 β is lower (Lucy, 2003, 2007). This is because tonic LH secretion is decreased, so the follicle will grow but not ovulate until the follicle reaches a size that produces sufficient estradiol to stimulate an LH surge. It is uncertain whether this low level of estradiol is due to increased metabolism of this hormone or problems with synthesis of estradiol by the follicle (Lucy, 2007). Low progesterone levels are hypothesized to result from a similar consequence in high producing dairy cows (Lucy, 2007). The lack of sufficient estradiol leads to many secondary effects including anestrus, persistent follicles, and multiple ovulations (Lucy, 2007). This may occur in part because of increased metabolism in high producing dairy cows due to increased hepatic blood flow, resulting in lower hormone blood levels than normal. This in turn may cause reproduction problems like multiple ovulations due to less negative feedback (Wiltbank, 2006). This decrease in blood estradiol concentration is also linked to shorter detectable periods of estrus.

The second category of infertility includes suboptimal or irregular estrus due to ovarian disease. An example is cystic ovarian disease, which differs from the above cause of anestrus. In this case LH levels are elevated in cows, and luteal function is abnormal including decreased progesterone production (Lucy, 2007).

The third category, abnormal pre-implantation development, is attributed to metabolic damage to oocytes in early lactation caused by release of nonesterified fatty acids from adipose tissue (Lucy, 2007). When oocyte quality is poor or early embryo development is stunted by stressors such as heat or compromised energy balance, embryo transfer may be used to increase pregnancy rates (Hansen, 2004; Lucy, 2007). Retrospective studies show

constant pregnancy rates for embryo transfer in dairy cattle over time (when recipients are not lactating dairy cows) despite the overall dairy cattle fertility decline (Hasler, 2006). This indicates that the quality of oocytes and resulting embryos has not been affected over time. Another supporting indicator is that fertility of non-lactating dairy heifers remains high, and only declines with lactation (Hasler, 2006).

The last category of infertility defined by Lucy (2007) was uterine or placental incompetence. This is associated with early embryonic loss during maternal recognition at 17-21 d of pregnancy, or placentation, at 4 to 6 wk of pregnancy. Only limited data are available for this aspect of historic fertility decline due to the relatively recent routine application of ultrasonography that has made identification of early pregnancy common (Lucy, 2007). Circumstances that contribute to early embryonic loss include breeding too soon postpartum, or diseases such as endometritis or mastitis that cause a suboptimal intrauterine environment for the developing embryo (Soto et al., 2003; Lucy, 2007). Loss of body condition appears to contribute to infertility in addition to the other causes just described.

Short term methods to improve fertility include synchronizing estrous cycles, ensuring that sires with high fertility are used, and use of rations that are not detrimental to reproductive hormonal activity (Lucy, 2007). In addition, an obvious aspect of management on an individual farm basis is optimization of herd health by improving nutrition to maintain a better energy balance, and decreasing infections of livestock or developing offspring (Stroud and Hasler, 2006).

Crossbreeding has also been proposed to improve fertility, but the potential decrease in milk production may make this option slow to evolve (Touchberry, 1992; Heins et al.,

2008). Long-term remedies include selective breeding for fertility, although this will slow the process for increasing milk yield

Synchronization of estrous cycles and ovulation of lactating dairy cattle eliminates the need to detect estrus by traditional means. Practitioners or technicians sometimes still monitor reproductive cycles of a herd, but also synchronize ovulation to allow mass breeding to maximize results. Gonadotropin-releasing-hormone (GnRH) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are administered in a series of three injections to synchronize follicular development, luteolysis and ovulation (Vasconcelos et al., 1999). This Ovsynch program is described in more detail in the Materials and Methods section of the thesis.

2. Assisted Reproduction

Sexing Sperm

Before reliable methods of separating sperm by sex were developed based on DNA content, numerous attempts to separate sperm to bias future embryo's sex were attempted (Garner and Seidel, 2008). These proved to be ineffective because of the unique characteristics of sperm. Mammalian sperm within males are essentially phenotypically identical (Seidel and Johnson, 1999), and approximately equal numbers of offspring of each sex result after insemination.

The technology of sexing sperm has many applications. These include averting situations in which sex-linked disease is a concern, family or endangered species gender balancing, increasing numbers of bull calves for beef production, or increasing heifer calves for dairy replacements (Lu et al., 1999; Seidel and Johnson, 1999; Wheeler et al.,

2006). As of 2005, at least 23 species of mammalian sperm had been sorted, with seven species producing normal-appearing offspring (Garner, 2006). In the case of dairy cattle, the heifer calf often is worth nearly three times as much to a dairy farmer as a bull calf (Wheeler et al., 2006); furthermore, she decreases the risk to the herd's overall health when used as a replacement relative to an outside purchase.

A heifer calf from a genetically superior cow more likely will be a superior milk producer than a replacement heifer from an unknown source. However, the dairyman's primary concern usually is the future milk production of the cow being inseminated, which means sperm must be sexed in a way that is not only rapid and efficient to make it commercially feasible, but also must be as non-damaging as possible to the sperm cells so that high fertility is retained. Furthermore, the expense of adding this technology to an already successful program would have to be less than the potential benefits. Additionally, development of this technology should consider the potential to lower the price and/or improve the quality of the final products, i.e. milk and meat (Seidel and Johnson, 1999).

Sex-sorting sperm initially was accomplished in 1981 from a collaboration between the Lawrence Livermore National Laboratory, Oklahoma State University, and the USDA. This sorting process involved nuclear treatment of sperm with DMSO, ethanol, proteolysis and DAPI stain (Garner, 2006), which was lethal to sperm (Garner, 2001; 2006). In 1989 the first repeatable method was developed for sexing live sperm (Johnson et al., 1989). Sperm were stained with a fluorescent DNA binding dye and run through a cell sorter. In the sorter, fluorescence was amplified to detect the small difference between X and Y chromosome bearing sperm. Bovine sperm with the X

chromosome contain 3.8% more DNA than Y sperm. Subsequently, modifications were made to improve the process such as quenching the fluorescence of dead or damaged sperm with food coloring (Garner, 2001) so that these sperm could be discarded. More rapid sexing ($5-6 \times 10^6$ sperm/h of each sex); (Wheeler et al., 2006) and more accurate measurement of fluorescence from the flat surface of the sperm nuclei (Garner, 2006) were made possible by beveling the sample injection tube tip and other changes to induce hydrodynamic orienting of individual sperm. By the year 2000, sexed sperm became available commercially in the United Kingdom (Garner, 2001), and several methods for quality control of separation accuracy became available including resort analysis, PCR and fluorescence in situ hybridization (FISH); (Seidel and Johnson, 1999; Garner and Seidel, 2008).

IVF using sexed sperm had initial success early on (Cran et al., 1994); however, abnormalities are higher than normal in offspring derived from IVF. Even without using IVF, it was critical to thoroughly examine the effects of sexing sperm on progeny (Seidel and Johnson, 1999; Garner, 2001). Increased early embryonic death has not been observed from using sexed sperm, but this has not been studied thoroughly. There was also no increase in late term abortions in cattle, relative to unsexed sperm, and calves appear to be normal (Tubman et al., 2004).

To summarize, the only effective means of separating X- and Y-sperm is by the difference in DNA content, identified when sperm are stained with Hoechst 33342 and sorted by using flow cytometry. Bovine X and Y spermatozoa have a 3.8% difference in DNA content. This results in 85-95% accuracy when sorting (Garner, 2001; 2006, Wheeler, 2006; Schenk and Seidel, 2007). Although accurate, this process is slow in

terms of sorting the number of sperm necessary for AI. It is also a costly process when considering the need for a \$350,000 machine, which with two nozzles produces only 10-20 doses of sexed sperm per hour. Using sexed sperm for IVF requires less sperm than AI, but typically results in low fertilization, cleavage, blastocyst and pregnancy rates (Lonergan, 2007; Lonergan and Fair, 2008)

Fertility is lower with sexed sperm than unsexed sperm because sperm are damaged mechanically and due to the time sperm are held for the sorting process (Seidel and Garner, 2002; Seidel and Schenk, 2008). This leads to a partial capacitation of sperm that shortens viability (Lu et al., 1999; Wheeler, 2006; Lonergan, 2007), and is exacerbated by cryopreservation (Seidel and Garner, 2002; Seidel and Schenk, 2008). With current sorting procedures, pregnancy rates usually are within 70-80% of unsorted sperm (Seidel and Garner, 2002). An additional problem is the tolerance of sperm to the sperm sorting process that varies between sires and individual ejaculates and is still poorly understood (Wheeler et al., 2006; DeJarnette, 2010).

Artificial Insemination

Timed AI in an estrus synchronization protocol is routinely used in the dairy industry, and sexed sperm is rapidly becoming widely used as well (Shenk et al., 2009; De Vries et al., 2008; De Jarnette et al., 2010). Routine AI of cattle using unsexed sperm is usually done with $15\text{-}20 \times 10^6$ sperm per dose. Sexed sperm typically is packaged at 2×10^6 sperm/straw, and even with this low number of sperm, fertility is 70-80% of control fertility (Seidel et al., 1999; Garner, 2001 and 2006; De Jarnette et al., 2010). When sexed and non-sexed sperm were inseminated in equal numbers, sexed sperm

pregnancy rates were 80-90% of non-sexed sperm (Seidel and Schenk, 2008; Schenk et al., 2009), but results from commercial use may be slightly biased due to producer selection of cows for sex-sorted sperm (DeJarnette et al., 2010). Some bulls have significantly lower pregnancy rates than others for sexed versus control sperm (DeJarnette et al., 2010). This decreased fertility may be remedied by increasing sperm numbers for particular bulls (DeJarnette et al., 2010) or may simply limit use of certain bulls for this technology.

For routine timed artificial insemination, cows are typically inseminated 16-18 hours after their final GnRH injection to fertilize the ovulated oocyte. It may be useful to postpone insemination with sexed sperm by a half day to insure that the oocyte is available to be inseminated (Lu et al., 1999; Seidel et al., 1999; Schenk et al., 2009) as small numeric increase in pregnancy rates occurred when insemination occurred a half day later than normal (Seidel et al., 1999). This has been proposed by many to be due to the additional handling of sperm during the sorting process that shortens sperm viability after thawing, which in part is due to the time that sperm are handled before freezing, and the force with which the sperm are sorted (Lu et al., 1999; Wheeler et al., 2006; Seidel and Schenk, 2008; DeJarnette et al., 2010).

Embryo Transfer

The first reported successful ET of a mammalian embryo was recorded in 1890, but the first reported calf born resulting from ET occurred 61 years later (Willett et al., 1951). The use of embryo transfer in commercial cattle breeding began in the early 1970s to increase the progeny of the few, valuable imported cattle at that time. This was partly due

to the introduction of nonsurgical embryo recovery and transfer techniques (Hasler, 2003, 2006; Seidel et al., 2003). Prior to that, most embryo transfer was done surgically under general anesthesia through a mid ventral incision, or under local anesthesia via a flank incision (Hasler, 2006; Seidel et al., 2003). By the mid 1970s nonsurgical transfer of embryos dominated the industry, and new forms of ART like embryo cryopreservation, embryo splitting, and creation of embryos by IVF were being applied in the dairy industry (Hasler, 2006; Seidel et al., 2003). The cryopreservation of embryos allowed for more flexible scheduling of ET and eliminated the need for maintaining large numbers of synchronized recipients for a particular batch of embryos (Hasler, 2003). Sexing embryos was also developed years earlier, by karyotyping of embryonic cells, but this was replaced by PCR in commercial practice due to the increased accuracy and decreased processing time (Seidel et al., 2003). Unfortunately, both methods require skilled technicians and special equipment, and therefore are quite expensive.

There were fairly steady numbers of Holstein embryo transfers from 1993 to 1999, which resulted in 30-36 thousand calves registered per year (Hasler, 2003). One issue with the use of ET in dairy breeds is the large number of bull calves produced that are of little value to dairy farmers. Using X-sorted sperm for dairy ET nearly eliminates production of the low value animals, and this dilutes the cost associated with purchasing sexed sperm. Y-sorted sperm could be used to produce bull calves for beef.

Through these advances, nonsurgical embryo transfer can be tailored to a particular farm's needs by combining technologies like sexed sperm, IVF and cryopreservation to allow the most flexible and economic choices when planning a reproduction program. Still, embryo transfer using IVP embryos, not to mention those produced with sexed

sperm, can be associated with problems that include decreased cryotolerance, changed expression of mRNA, lower pregnancy rates, and increases in abortion rates as well as fetal and neonatal abnormalities (Hasler, 2003; Lonergan and Fair, 2008). This may be translated into commercial practice where the majority of ET has been done with in vivo-derived embryos, over half of which are transferred frozen. Fewer in vitro-produced embryos have been transferred worldwide than in vivo embryos, with the majority being transferred fresh (Lonergan and Fair, 2008; Thibier, 2009).

Some benefits of embryo transfer include protecting against disease transmission, maximizing embryos per semen dose, and increasing pregnancy rates in certain circumstances. If problems associated with IVF could be ameliorated, then convenience and efficiency could be added to the list, particularly when considering using sexed sperm in the system (Lonergan, 2007). The zona pellucida is considered a barrier for the embryo against viral infection, and when washed properly, per the Manual of the International Embryo Transfer Society guidelines, the risk for transmitting disease from the embryo to the recipient cow is essentially eliminated (Seidel et al., 2003). This is true for animals in general as there is sparse evidence of infectious agents including endogenous retroviruses that are transmitted vertically from parent to offspring. This is also true for transmissible spongiform encephalopathies (Wrathall and Suttmöller, 1998).

Embryo transfer often is used to circumvent reproduction problems. If a cow has adhesions of the upper reproductive tract that cause problems producing embryos, a healthy embryo can be transferred to develop in her uterus if the endometrium is healthy and hormone levels are normal. Likewise, if there is a problem maintaining a pregnancy due to permanent damage from a uterine infection, normal embryos may be able to

develop to term if transferred to younger or healthier recipients to preserve the genetics of a valuable donor (Seidel et al., 2003).

3. In Vitro-Production (IVP) of Bovine Embryos

It is well known that the two main components of IVP of bovine embryos are the oocyte and the sperm. What may not be as obvious is the importance of each intricate detail in the IVF process, from the collection and preparation of the sperm and oocytes, to the preparation of the environment for fertilization, to the method of fertilization as well as embryo culture. Each of these aspects of IVP of embryos could benefit from further investigation due to the fact that pregnancy rates after transfer of IVP embryos are lower than those of in-vivo derived embryos (Loneragan, 2007).

Quality Control of IVP Bovine Embryos

In vitro fertilization of mammalian oocytes is a complex process that involves careful manipulation of the gametes (sperm and oocyte) and the environment to produce embryos. Items to consider include culture media composition, atmospheric gasses, temperature, and light exposure. These must be optimized for the species of interest; for example, bovine gamete and embryo culture has greater success at warmer incubation temperatures than those for culturing mouse embryos (Gardner, 2004). Failure to recognize and cater to the developing zygote's evolving requirements can result in altered gene expression, a halt in embryo development at a certain stage, and reduced blastocyst formation, leading to an overall decrease in viable offspring (Schiewe, 1998; Preis et al.,

2006; Nagy et al., 2003; Gopichandran and Leese, 2006; Barcelo-Fimbres and Seidel, 2007). In vitro-produced embryos also have an increased sensitivity to cryopreservation and lower post transfer success rates when compared to in vivo-developed embryos (Barcelo-Fimbres and Seidel, 2007).

Culture media not only require particular nutrients for embryo development, but also need to be adjusted to physiologic osmolarity, gas concentrations, and pH. An example illustrating such specificity is the bovine IVP protocol of De La Torre et al. (2006a), in which the osmolarity of the fertilization medium is around 300 with a pH between 7.5-7.6 and is equilibrated to 5% CO₂ and in air, while CDM-1 medium for early embryonic development has an osmolarity of around 275 with a pH between 7.3-7.4, and is equilibrated to 5% O₂, 5% CO₂, 90% N₂ in a tri-gas incubator. Oxygen concentration of 5% is vastly superior to the oxygen concentration in air, 21% (Olson and Seidel, 2000) for culturing embryos; however, when oocytes are maturing and being fertilized, oxygen concentrations are less crucial than during subsequent culture to blastocysts (Lonergan, 2007). Oocytes, sperm and developing embryos must be incubated at the body temperature of the species being used for optimal development. For bovine IVF, it is ideal to keep embryos at 39°C, although due to thermometer differences it may be safer to incubate at a 0.5°C lower temperature. In addition, direct sunlight and cool-white fluorescent light can be detrimental to developing embryos (Schiewe, 1998; Takenaka et al., 2007). Light filters can be used to circumvent this problem. Materials that come into contact with the embryo must be sterile and toxin-free. Chemicals from sterilization like ethylene oxide and benzothiazoles found in some syringe plungers are toxic to embryos (Schiewe, 1998).

One problem with in vitro culture of embryos is mimicking the variable oviductal and intrauterine environment that occurs during the reproductive cycle. Various factors for communication between the reproductive tract and the embryo are missing in vitro. (Gopichandran and Leese, 2006; Vajta, 2008). With in vitro culture, the objective is to provide the embryo with optimal nutrients and environmental control while considering the practicality of a system that will work consistently at reasonable cost and effort. One means to circumvent the lack of maternal mediators is to co-culture embryos with somatic cells of the reproductive tract. This improves development rates and cell numbers, but can result in abnormal pregnancies (Lane et al., 2003). Another concept is to culture multiple embryos in close apposition to each other. This improves developmental rates by stimulating cell division and limiting apoptosis through paracrine or autocrine mechanisms (Gopichandran and Leese, 2006).

Antibiotics are used in culture and sterile laboratory conditions are used in lieu of the immune system. Although sterile technique is practiced, embryos are exposed to unfiltered air when culture media are changed or when embryos are sorted and washed (Stringfellow, 1998; Riddell and Stringfellow, 1998). Common antibiotics used to prevent microbial growth during IVP of embryos include gentamycin, amikacin, streptomycin and penicillin. Often bacterial contamination is introduced through the sperm or is harbored in cumulus cells surrounding the oocyte during aspiration. Contamination can also occur from handling violations such as reaching over dishes or media or talking while handling exposed embryos.

More specific information for embryo culture and quality control issues is provided in the Manual of the International Embryo Transfer Society. This internationally

recognized publication addresses quality control issues including pathogen control and international requirements for importing and exporting embryos. There are also many specific tissue culture manuals that provide detailed information on quality control and other aspects of embryo culture, for example, *A Laboratory Guide to the Mammalian Embryo* by Gardner et al. (2004).

Oocytes

Oocytes used for bovine IVP embryos can be obtained by transvaginal aspiration, but far more oocytes can be obtained in a shorter period of time, and at a considerably lower cost by harvesting cumulus-oocyte complexes (COC) from ovaries collected from an abattoir. It is common to aspirate follicles between 3 and 8 mm in diameter (Lu et al., 1999; Park et al., 2005) that contain ideal COC with more than three layers of cumulus cells and evenly granulated cytoplasm (Lu et al., 1999; Park et al., 2005). Development of IVP embryos from abattoir derived oocytes that are matured in vitro are not as good as oocytes matured in the ovary in vivo. Blastocyst rates for oocytes matured in vitro are generally 15-40%, while in vivo matured oocytes yield 50-80% blastocyst per oocyte (Park et al., 2005). Some experiments with growth factors may produce over 50% blastocyst rate per oocyte but these results are inconsistent (Lonergan, 2008). The discrepancy in blastocyst rate between in vitro and in vivo-matured oocytes is likely explained by the accelerated maturation of in vitro-matured oocytes and the inability to mimic the follicular environment. Normally, in vivo follicles that ovulate contain mature, metaphase II oocytes; the follicles are 12-18 mm in diameter and have been allowed to mature days longer than in vitro matured oocytes (Lonergan, 2008). Accelerated

maturation time in vitro is coupled with the lack of the positive signal for maturation by the LH surge that occurs in vivo, and may be why cytoplasmic maturation is incomplete (Lonergan, 2008; Panigone et al., 2008). Oocytes and cumulus cells in smaller follicles are not directly stimulated by LH due to the lack of LH receptors (Panigone et al., 2008). The Oocyte quality used for IVP embryos can be the most significant factor affecting embryonic development when all other conditions are equal (Lonergan, 2008).

Timing of oocyte maturation is important because it is linked to later development. A commonly described maturation time for oocytes is around 23 h (Lu et al., 1999). Notably, a shorter rather than longer incubation time for oocyte maturation, before fertilization, is preferred. Oocytes should be at a stage where the first polar body is extruded but they should not be aged too far beyond this point (Park et al., 2005). An optimal oocyte maturation protocol would result in the highest percentage of oocytes developing to competent blastocysts that result in viable offspring after transfer and gestation (Park et al., 2005).

One problem with abattoir-derived oocytes is the lack of history of individual oocytes including stage of development and donor information (Lonergan and Fair, 2008). This usually makes the genetics of the oocytes unknown. Commercially available bovine oocytes that are aspirated and shipped during the maturation process can be ordered with particular breeds requested. Little other information is available for these oocytes, and even the breed information sometimes is incorrect. In addition, it is difficult to verify the reliability of the oocyte company until calves are born, which would be problematic for a carefully controlled reproduction program.

When culturing oocytes for maturation and producing embryos after fertilization, it is important to understand that the initial conditions could greatly affect subsequent embryo quality. It is suggested that the most critical culture step to create quality embryos is maturation of the oocytes, although it appears that removal of an oocyte from a small follicle limits the oocyte's developmental potential (Rizos, 2003; Lonergan and Fair, 2008).

Sperm

Frozen-thawed sperm are capacitated, generally by centrifugation either through medium or a percoll suspension, and then incubation with heparin (Parrish, 1989). When thawing sperm, it is important to protect sperm from UV and temperature shock. Sexed sperm, due to the previous processing are particularly vulnerable to environmental effects (Garner, 2006; Schenk et al., 2008).

Processing sperm whether for cryopreservation, sexing or IVF damages sperm cells. Sperm are normally in seminal plasma that contains various components for energy and stabilization of membranes (Maxwell et al., 2007). When sperm are processed they are exposed to solutions that can cause a dilution effect by displacing stabilizing proteins that prevent the acrosome reaction (Maxwell et al., 2007). This pushes sperm toward a capacitated state that shortens the lifespan of sperm cells (Maxwell et al., 2007). Capacitated sperm cells utilize available energy more rapidly during membrane destabilization and hyperactivation (Alomar et al., 2006). Sire differences in response to treatments is multifactorial in that the content of seminal plasma that directly affect the hardiness of sperm cells can vary with nutrition, temperature, stress and season (Maxwell

et al., 2007). This may help understanding the conflicting results of some studies regarding bull effects (Zhang et al., 2003; Xu et al., 2006; Schenk et al., 2009). Additionally, effects of exposure time to and components of seminal plasma are not completely understood (Maxwell et al., 2007).

4. Blastocyst Production and Relative Stages of Development

In vitro-produced embryos typically result in low fertilization, cleavage, and blastocyst rates (Lonergan and Fair, 2008), and the embryos are inferior to those produced in vivo (Lonergan, 2007). Several factors in the success of IVP embryos have been discussed and include variables in gamete quality, handling and in vitro culture conditions at each stage of embryo development, (Lonergan, 2007; Lonergan and Fair, 2008). Even when a particular IVP method is optimized, by minimizing sperm variability and using reliable suppliers, quantitative results can still vary considerably due to oocyte batch differences (Alomar et al., 2006; Lonergan and Fair, 2008). This is also true for the performance of an individual sire's sperm. Cryopreservability of a bull's sperm and subsequent fertility may vary by ejaculate (Maxwell et al., 2007). Prospective bulls are best evaluated for their sperm's ability to fertilize oocytes in vitro before being chosen for use on a large scale. Pregnancy rates would be the best method of evaluation of IVF success, but more practical methods of selection such as fertilization and blastocyst rates are generally used. It is not completely clear how to interpret cleavage and blastocyst rates as indicators of how well a bull's sperm may perform when used for IVF. Some studies have shown a correlation of cleavage to blastocyst rates to be important indicators

of fertility while other studies have not shown a predictable correlation (Alomar et al., 2006). However, when using sexed sperm for IVF, selection of a particular bull's sexed sperm that results in acceptable cleavage and blastocyst rates was more important to predict pregnancy success than when using unsexed sperm (Wheeler et al., 2006). AI pregnancy rates using X-sorted sperm may give an idea of how tolerant a particular bull's sperm are to the sexing process. However, AI pregnancy rates may likely be independent of how well a bull's sperm will work for IVF or IVF using sexed sperm. Another possible confounder when predicting the success of a bull's sperm for IVF, after embryos have been transferred, is considering how an individual farm's management can affect the fertility of the recipient cows.

Typical blastocyst production rates per oocyte are 30-40% for non-sexed sperm and 10-20% for sexed sperm (Wheeler et al., 2006; Lonergan, 2007). Embryos produced with sexed sperm are slightly delayed in development after the first cell cycle (Lu et al., 1999; Wheeler et al., 2006). This could be due to chromosomal damage during the sorting process, Hoechst 33342 binding to DNA, or culture conditions not tailored to slightly retarded first cell cycle development. Embryo development may improve as the sexing process is improved. Whether or not these conditions can be remedied, considering that sperm must be passed through the sorter over time to make this technology practical (Seidel and Garner, 2002) has yet to be determined.

5. Success Rates with IVP Embryo Transfer and Calving in Cattle

IVP embryos result in lower pregnancy rates than those that develop *in vivo*. Some problems are less compaction of morulae, fewer cells in the inner cell mass, retarded development, and sex ratios skewed toward males (Hasler, 2003). Pregnancy rates for *in vitro*-produced embryos average around 20-40% while those of *in vivo*-embryos are 50-60% (Park et al., 2005).

Calves produced with sexed sperm appear normal (Seidel et al., 1999). In an early field trial of insemination of heifers with sexed sperm, there were no differences in gestation length, abortions, calving problems, weight of calves or calf vitality compared to those calves produced from insemination with unsorted sperm (Seidel et al., 1999; Hasler, 2003). Tubman et al. (2004) had similar findings. Some common abnormalities such as large offspring syndrome and problems at parturition have been linked to IVP embryos independent of using sexed sperm (Hasler, 2003). Large offspring syndrome is characterized by abnormally large offspring and extended gestation lengths. Calves can have enlarged or malformed organs such as umbilical cords, muscle, nervous tissue, and/or bone. In addition, calves can have pathologic metabolic processes that may cause early fetal loss or be present in the neonate (Young et al., 1998; Lazzari et al., 2002). These developmental abnormalities are often linked to particular culture conditions or media ingredients for example co-culture of embryos with somatic cells or addition of fetal calf serum to the medium (Hasler, 2003; Lonergan and Fair, 2008; Park et al., 2005). These conditions can stimulate accelerated growth and cause persistent growth abnormalities (Young et al., 1998; Lazzari et al., 2002). However, further analysis of

even larger numbers of calves born from sexed sperm needs to be done to determine if there are subtle or less common genetic abnormalities.

III RESEARCH PROJECT

Introduction

Fertility of dairy cattle declined markedly between 1975 and 2000 (Lucy, 2001), and remains low. This is true for cows inseminated by AI as well as by natural service. In light of the current fertility issues, approaches complementing or substituting for AI could theoretically be quite advantageous (Hansen, 2003). For example, pregnancy rates following transfer of fresh embryos are substantially higher than those following AI in heat stressed cattle (Hansen and Block, 2004). Another benefit of using ET would be to decrease the twinning rate.

In addition to the benefits to dairy cow fertility, ET can make other technologies more efficient. For example, transfer of embryos produced *in vivo* or *in vitro* with sexed sperm is an attractive idea for heifer calf production. The use of sexed sperm to produce embryos should not affect normality of calves compared to those produced with non-sexed sperm. Evidence for this is that normality of calves produced by artificial insemination with sexed sperm did not differ from calves produced with non-sexed sperm (Tubman et al., 2004).

The added expense of trained personnel and the lower embryo production rates with sexed sperm may make this approach impractical for some cattle operations. Herds with particular fertility problems or that have planned expansion should consider the benefits of applying these technologies to their reproduction program (Hansen and Block,

2004). This study was designed to determine the practicality of ET and AI using sexed sperm on commercial dairy farms.

Materials and Methods

In Vitro Fertilization and Culture Media

All reagents for preparing media were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. TCM-199 with Earles salts +0.5% fatty acid-free BSA (Sigma # A-6003) with 25 ug/ml gentamycin sulfate was used to mature cumulus-oocyte complexes (COC). Media for IVF and embryo culture were the series F-CDM (fertilization), CDM-1 (early embryo culture), and CDM-2 (later stage culture) as described by De La Torre et al. (2006a), which also included H-CDM-1 and -2 that had 25 mM hepes and 5 mM bicarbonate to maintain pH in air during brief embryo handling during the vortexing procedure of presumptive zygotes, and examination of cleavage and blastocyst formation.

Commercially available frozen, sexed and non-sexed sperm from three Holstein bulls for Dairy 1 (2 from XY Inc., Fort Collins, CO; 1 from Select Sires, Plain City, OH) and two of the same bulls for Dairy 2, were used for either artificial insemination or for in vitro fertilization with purchased grade Holstein oocytes (BoMed Inc., Madison, WI). At each dairy, embryos produced with sexed or unsexed sperm were transferred fresh. Four methods to produce pregnancies were tested: (1) AI with unsexed sperm (control); (2) AI using sexed sperm; (3) embryo transfer using embryos produced using unsexed sperm, transferred fresh; and (4) embryo transfer using embryos produced with X-sorted sperm,

transferred fresh. In addition a few embryos produced with sexed sperm were frozen, thawed and transferred. Results for this treatment were so poor that this treatment was dropped. (See Appendix 1)

Syngro® medium, straws and plugs were purchased from, and 6 well manipulation plates were donated by Bioniche Life Sciences AB Technology (Pullman, WA); embryo manipulating pipette tips with filters were purchased from Life Science (St. Petersburg, Fl). Lidocaine, needles and syringes were purchased from Mountain Vet Supply (Fort Collins, CO).

This experiment consisted of 4 treatments using 3 bulls for Dairy 1 and 4 treatments using 2 bulls for Dairy 2.

Embryo Production

Embryos were produced using standard IVF/IVP techniques with specific deviations described below. To F-CDM media, 10 ug/ml heparin and 20 ug/ml amikacin (in addition to the 25 ug/ml of gentamycin sulfate) were added for both sexed and control sperm IVF procedures. The CDM-2 culture medium contained 0.25 uM phenazine ethosulfate (PES) to decrease lipid content of embryos (De La Torre-Sanchez et al., 2006b). All fertilization and culture occurred under sterile paraffin oil (7.5 ml for the drop system in 60 mm petri dishes and 250 ul per well in the 4 well system). All culture and wash dishes with media were equilibrated (38.5°C, 5% CO₂, 5% O₂, 90% N₂) at least 4 h prior to adding gametes or embryos.

The laboratory received 250 Holstein COC weekly for Dairy 1 and 150 per week for Dairy 2. The COC were shipped overnight from the company BoMed in Wisconsin in

a portable incubator (Minitüb GMBH, MT 35/42-'92) and cultured in 2 ml vials with 50-75 COC per vial. Upon arrival, the temperature of the portable incubator was checked to be within 37-39°C before placing COC into the laboratory incubator (38.5°C, 5% CO₂ in air) in the shipping vials with lids loosened, until 23 h of maturation had elapsed.

When oocyte maturation was complete, all COC were transferred in 10 ul of maturation media and washed in a new well with 500 ul of F-CDM. The COC were randomly assigned to be inseminated with X-sorted sperm or non-sorted sperm before the final transfer into F-CDM medium for IVF.

All sperm were thawed, no more than two straws at a time in a 37°C water bath for 30 s. Control sperm were placed on top of a standard percoll gradient (2 ml 45% percoll over 2 ml 90% percoll) in a 15 ml conical tube, centrifuged for 20 min at 600X g, and supernatant removed to just above the pellet. The sperm were re-suspended and washed in 4 ml of H-CDM-1 by centrifugation for 10 min at 600X g. The sperm concentration was determined using a hemacytometer, and the cells were diluted to 5x10⁶ sperm/ml. Fertilization of COC with control sperm was done in four well dishes with 440 ul of IVF media, 10 ul COC in F-CDM and 50 ul of sperm suspension for IVF.

Sexed sperm were prepared using a mini-percoll gradient (500 ul 45% percoll over 450 ul 90% percoll) in a 1.5 ml conical tube. After X-sorted sperm were thawed, they were layered on top of the mini-percoll gradient, the 1.5 ml tube was capped, and the suspension was centrifuged at 600X g for 10 min. The supernatant was removed leaving a pellet and approximately 50-100 ul of fluid. One ml of H-CDM-1 was then added to wash and re-suspend the pellet. Then the tube was centrifuged at 300x g for 5 min. Supernatant was again removed to leave a pellet. Sperm concentration was then

determined and the concentration was adjusted to 10×10^6 sperm/ml before the final dilution into the fertilization drop resulting in 2×10^6 sperm/ml. The fertilization drop consisted of 30 ul of the sexed sperm, 10 ul COC, and 110 ul F-CDM under oil in 60 mm dishes. Small drops of medium were used for fertilization with sexed sperm due to limited number of sexed sperm per straw.

Sperm and oocytes were co-incubated for 18 h at 38.5°C with a 5% CO_2 in air atmosphere. After a visual motility check of the sperm, COC were placed in H-CDM-1 to vortex in 0.5 ml snap top tubes for 1 min to remove cumulus cells. Immediately after exposure to the vortex, oocytes were washed from the sides of the tube and suspended in 200-400 ul of H-CDM-1. After the oocytes settled in the tube, a 7-drop wash system was used to separate oocytes from debris and loose cells. After visible debris was removed, approximately 50 presumptive zygotes were cultured per 4 well dish in 500 ul of CDM-1 (incubated at 38.5°C , 5% CO_2 , 5% O_2 , 90% N_2).

Approximately 56 h after the vortex treatment, embryos were checked for cleavage and development status. Uncleaved oocytes were discarded. Embryos that had developed to 2-4 cell stage were retarded and separated from the more advanced normal-appearing embryos consisting of six or more cells. All embryos were cultured for an additional 4.5 d in CDM-2 at 38.5°C , 5% CO_2 , 5% O_2 , 90% N_2 .

Embryos for transfer were placed in Syngro® and loaded into 0.25 ml ET straws (Bioniche, ETE063). Embryos used for transfer (~7.5 d after onset of fertilization) were Grade 1 or 2 blastocysts, expanded blastocysts or rarely, early hatching or hatched blastocysts. Embryos were loaded into ET guns, covered with a 21 in plastic chemise (Bioniche, ETE030), and then put into gun warmers (EM Tools Inc., Rusk, TX) at 38°C ,

or kept at that temperature until a free gun was available. Embryos were transferred into recipients that were assigned to treatments by random selection.

Cryopreservation of Embryos

Some embryos fertilized for Dairy 1 were frozen in glycerol with an experimental direct transfer method. Those embryos were moved to Syngro® holding medium before transferring to a Glycerol freeze medium (GLY) (Syngro® holding with 0.1% PVA added to make a 0.2% PVA base medium plus 0.1 M sucrose and 1.5 M glycerol). A 0.5 M galactose dilution medium (GAL) (Base medium plus 0.5 M galactose) was loaded into straws for a post thaw dilution of the GLY. Straws were marked at 3, 3.5, 4.5 and 5 cm from cotton plug to ensure accurate amounts of each solution. Embryos were placed in the GLY freeze media for 10 to 20 min before beginning the freezing process. GAL was loaded into the straw to the first mark. Air was filled to the next line before loading the embryo in the 5 mm column GLY medium. After the second 0.5 cm air space, GAL was used to load the rest of the straw and seal the cotton/PVC plug. The open end of the straws was plugged with a plastic 0.25 and 0.5 ml convertible plug. A 0.5 ml straw was attached to serve as a label.

Straws were positioned horizontally until being loaded into the freezing machine (Freeze Control®, CL-3300). Cooling was performed as follows: Straws were loaded into the -6°C freezing chamber in pairs with the cotton plug side oriented down. After 2 min at this temperature straws were seeded at the first visible meniscus from the plastic plug. Straws were held for an additional 8 min before cooling at 0.5°C/min to -32°C. Straws were then plunged into liquid nitrogen for storage.

Embryos to be transferred were thawed on the farm in a 38°C water bath, shaken to mix compartments, loaded into the ET gun, and equilibrated 2 min in the gun warmer before transfers were performed.

No frozen IVF embryos were transferred at Dairy 2, but excess embryos were frozen as follows: embryos were moved to Syngro® for holding/rinsing before loading into either ViGRO™ Ethylene Glycol Freeze Plus 0.1 M Sucrose (EVM234; Bioniche, Pullman WA) or glycerol freeze medium. The glycerol freeze medium was as described above, but in this case, no galactose was used to load the straws. Each freezing replicate performed was designed to freeze similar quality and stage embryos from each treatment. These additional embryos were retained for future experimentation.

Animals

Cows used in this investigation were from two commercial dairies. Work at the first dairy (8,000+ cows) was done from August to December of 2007, and cows at the second dairy (800+ cows) were used between January and May, 2008. Only first service, lactating Holstein cows in an Ovsynch program were used. Cows at Dairy 1 were housed in dry-lot pens; at Dairy 2 cows were in dry-lot pens and covered free stalls with clean sand bedding. Cows at both dairies were milked three times per day and fed total mixed rations.

Estrous cycles of cows at least 60 d post partum (n=354 for Dairy 1 and n= 184 Dairy 2) were synchronized using Ovsynch procedures (Vasconcelos, 1999) as follows: ovaries and reproductive tracts were scanned using ultrasonography, and 100 ug GnRH was injected i.m. to the cows without apparent ovarian or uterine pathology. Seven d later

cows were scanned and given 25 mg of PGF2 α i.m. only if a CL or a follicle 13-19 mm in diameter was present on the ovary; 2.5 d later cows received 100 ug GnRH i.m. If structures were not observed, cows were restarted with GnRH with or without a CIDR, and some were eventually cycled back into the experiment. Cows were artificially inseminated 18 h after the second GnRH injection with control or sexed sperm, or cows were retained as recipients for a fresh, (or frozen for Dairy 1), embryo one week later. Embryos were transferred approximately 184 h from when oocytes were fertilized. Recipients' ovaries were scanned for a corpus luteum (CL) with quality and side noted; those without a detectable CL were not used. To relax rectal muscles of recipient cows for embryo transfer, cows were injected epidurally with 5 ml 2% lidocaine. Transfer of a single embryo was made into the uterine horn of each cow ipsilateral to the CL. Quality of the embryo transfer was also noted by the technician (1=normal, 2=slight difficulty, 3=problems such as cow lurching when the embryo was deposited, and 4= not likely to have transferred due to, for example, a bent straw).

Pregnancy status was determined at d 35 (Dairy 1) or 32 (Dairy 2) of pregnancy by ultrasound. Cows with dead or dying fetuses (at day 32 or 35) were considered not pregnant. Fetal deaths were determined between first check and a second check about d 60. Cows that died before respective pregnancy checks were excluded from data at those time points. Cows with pyometritis when checked for pregnancy were included in data (n=5 at Dairy 1) as that condition could relate to treatment. Cows losing pregnancies after d 60 were determined from herd records, and data were recorded at calving.

Experimental Design

There were 16 visits to Dairy 1 and 15 visits to Dairy 2. For Dairy 1, each day an average of 12 cows were randomly assigned an AI treatment, and 15 to ET. AI cows were treated with either sexed or non-sexed sperm. ET cows were assigned a fresh embryo fertilized with sexed or non-sexed sperm. Each treatment was done using three different bulls at Dairy 1. For Dairy 2, an average of 7 cows for AI and 6 cows for ET were used each day, but only two bulls were used.

Statistical Analyses

Cleavage rates and 7-8 day blastocyst rates were calculated for IVF. Treatment effects were analyzed by ANOVA; percentages were arc sin transformed prior to analysis. Values presented are untransformed least squares means \pm SEM. The model included treatments, bulls and their interactions. Pregnancy rates (1= pregnant; 0= not pregnant) by treatment and bull were analyzed by the Gen Mod procedure in SAS with technician and stage of embryo in the model in addition to bull and treatments.

Results

IVP Embryos

Overall cleavage rates for embryos produced with X-sorted (67%) and unsorted (69%) sperm were not different (Table 1); however, the cleavage rate for sexed sperm of Bull C (44%) was lower than for the control, unsexed sperm (69%); ($P < 0.05$) (Figure 1). Blastocyst rates for sexed sperm averaged over all bulls were less than rates for control

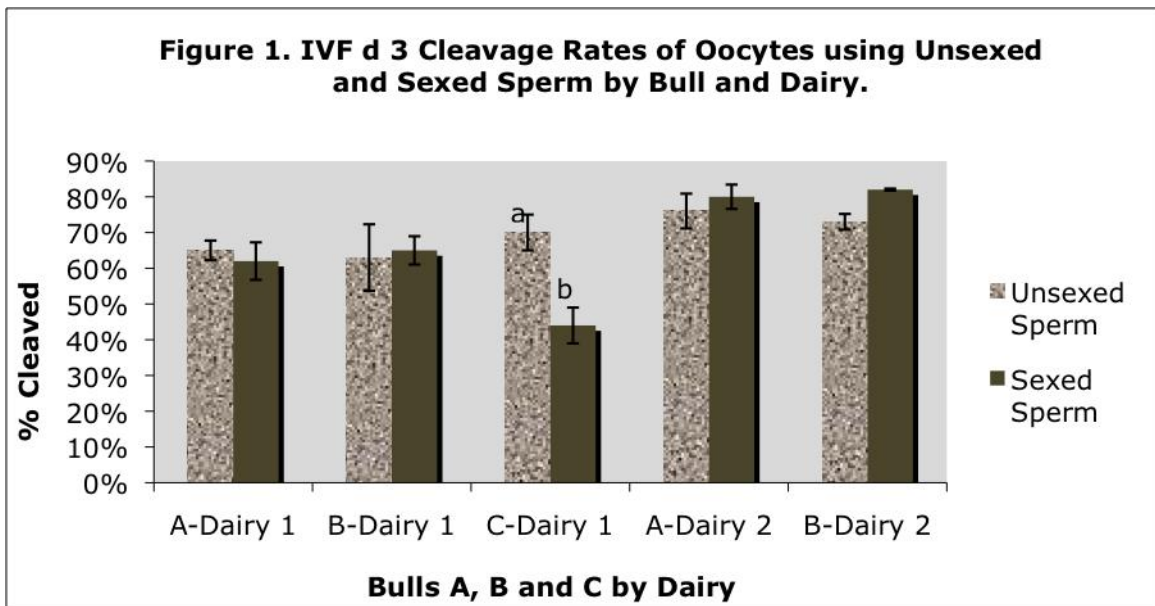
sperm ($P < 0.05$); (Table 1). The blastocyst rate for sexed sperm of Bull C was not significantly lower ($p > 0.1$) than for sexed sperm of the other bulls (Figure 2).

Table 1. Effect of sperm type on cleavage rates at day 3 and blastocyst development at day 7.5 following insemination.

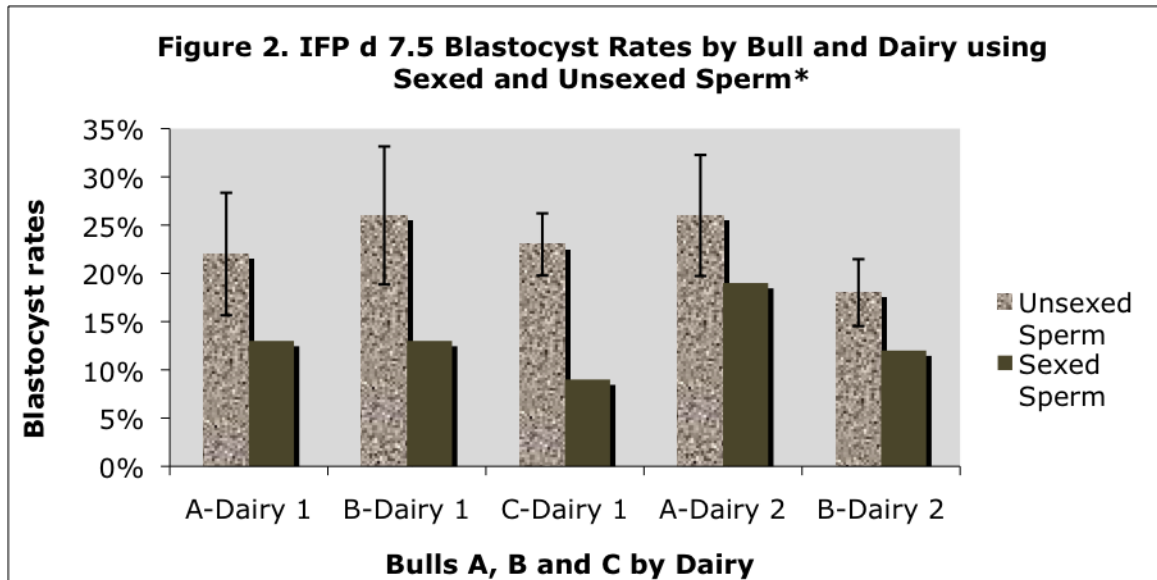
Sperm Type	# Oocytes	% Cleaved d 3	% Blastocysts d 7
Non-sorted	1785	69 ± 2.2	23 ± 2.3^a
X-sorted	3985	67 ± 3.1	13 ± 1.2^b

Data are least-squares means \pm SEM.

a,b means differ, $P < 0.05$.



a, b $P < 0.05$



* No significant differences among bulls
P < 0.05 difference between Control and Sexed sperm

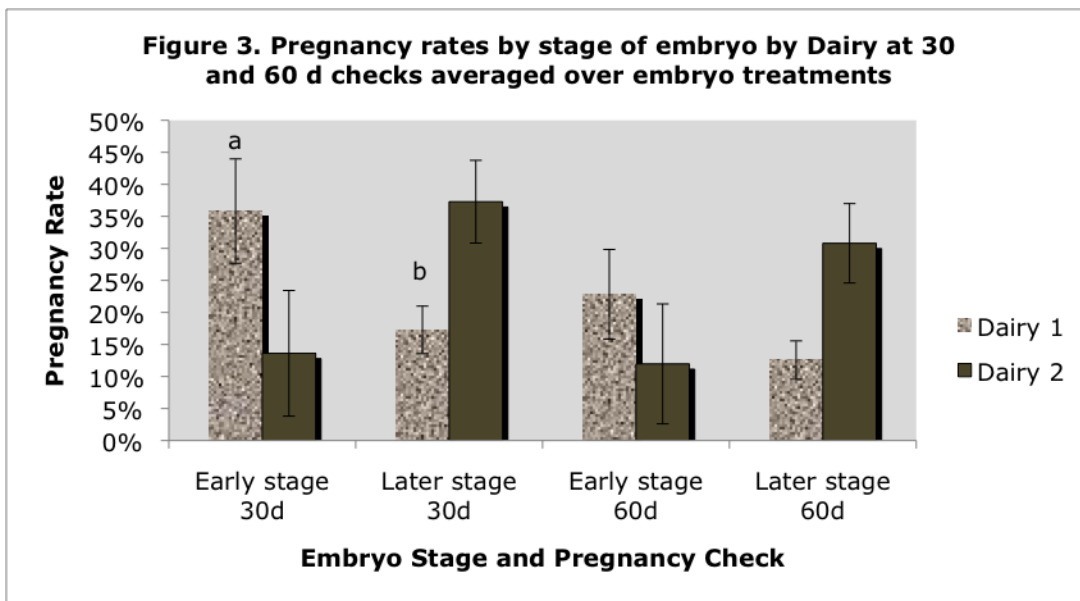
Transfers and Inseminations

At Dairy 1, 346 first service cows with healthy reproductive tracts, determined by ultrasonography, were selected for the project. Before treatments were administered, 32 cows (9.2%) were excluded from the project due to complications: Vaginal abscesses-2, pyometritis-7, recipient for ET had no or poor corpus luteum after OvSynch protocol-8, not found the day of ET-5, sick in hospital-3, in heat on the day of ET-1, cows that were treated but died before first pregnancy check-6. Cows with pyometritis at the 35 d pregnancy check were included and totaled five. Cows that had a dead conceptus at the time of first pregnancy check were considered not pregnant, which occurred 8 times.

Cows at Dairy 2 were selected in the same manner; 194 cows were selected with 9 excluded (4.6%). These included: recipient had no or poor CL after OvSynch protocol-2, in heat in the day of ET-1, cow was in poor condition per dairy's discretion-2, cervical

adhesions-2 and cow death-2. Cows with dead conceptuses at the first pregnancy check occurred 2 times and were considered not pregnant.

For statistical analyses embryos were grouped into two categories: early development (prior to the expanded blastocyst stage) and later development (expanded and hatching blastocysts). At Dairy 1 there was a significant difference between embryos of each group ($P < 0.05$); the earlier blastocysts had higher pregnancy rates at the 35 d check than later stage embryos; at the 60 d check there was no significant difference ($P > 0.05$) (Figure 3). For Dairy 2, earlier stage blastocysts rates were not significant ($p > 0.5$).



a, b $P < 0.05$
 (Early stage- prior to expanded blastocyst and Later stage- expanded through hatched blastocyst).

Pregnancy Rates by Treatments

For Dairy 1, AI pregnancy rates with unsexed and sexed sperm were not significantly different at the 35 d (47% and 41%) or 60 d (43% and 34%) pregnancy check (Table 2). At the 35 d pregnancy check pregnancy rates for embryos produced with unsexed and sexed sperm were similar to each other (23% and 19% respectively), but lower ($p < 0.05$) than AI with unsexed sperm (Table 2). Four cows died between the 35 and 60 d check. At 60 d, AI pregnancy rates with unsexed sperm remained higher than both ET groups (unsexed-22% and sexed-7%), but was not significantly higher than AI with sexed sperm. However, pregnancy rates for embryos produced with sexed sperm were lower ($p < 0.05$) than those for AI with and without sexed sperm (Table 2).

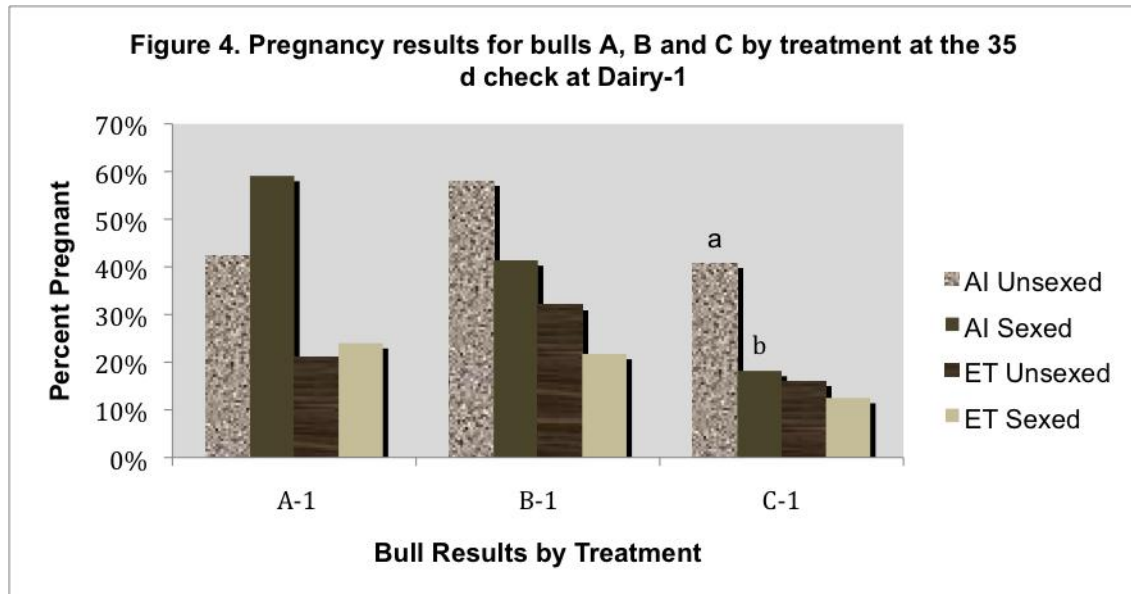
Table 2. Dairy 1-Comparison of pregnancy rates with embryos produced with non-sexed or sexed sperm and timed-artificial insemination with non-sexed or sexed sperm.

Treatment	N	35 d Pregnancy	N	60 d Pregnancy	% Calved
AI Unsexed Sperm	89	47% ^a	88	43% ^a	37% ^a
AI Sexed Sperm	84	41% ^{a,b}	82	34% ^{a,b}	26% ^{a,b}
ET Unsexed Sperm	69	23% ^b	68	22% ^{b,c}	18% ^{b,c}
ET Sexed Sperm	72	19% ^b	72	7% ^c	4% ^c

a,b,c Means within columns without common superscripts differ, $P < 0.05$.

Pregnancy rates did not differ significantly among bulls for unsexed sperm AI or either ET treatment, but bull C-1 had the lowest pregnancy rates for each of the treatments. Bull C-1 had significantly lower pregnancy rates when using sexed sperm verses non-sexed sperm (Figure 4.). Also the pregnancy rate for AI with sexed sperm for

Bull C-1 was lower than that of the other bulls ($P < 0.05$) (Figure 4.). This Bull's sperm was not used at Dairy 2 due to its poor performance.



a,b $P < 0.05$, Bull C-1 also had a lower AI pregnancy rate with sexed sperm than other bulls, $P < 0.05$.

For Dairy 2 at the 32 d check there was no significant difference in pregnancy results for transfer of embryos produced with unsexed or sexed sperm and artificial insemination with unsexed or sexed sperm ($P > 0.05$) (Table 3). At day 60 the pregnancy rate for ET using embryos produced with unsexed sperm was lower than the AI pregnancy rate using unsexed sperm ($P < 0.05$). Bulls did not differ significantly within the same treatment group (data not present). There was no significant difference between technicians for embryo transfer pregnancy rates at either dairy ($P > 0.1$).

Table 3. Dairy 2-Comparison of pregnancy rates with embryos produced with non-sexed or sexed sperm and timed-artificial insemination with non-sexed or sexed sperm.

Treatment	N	32 d Pregnancy	60 d Pregnancy	% Calved
AI Unsexed Sperm	44	46%	44% ^a	41% ^a
AI Sexed Sperm	62	40%	34%	31%
ET Unsexed Sperm	39	26%	21% ^b	15% ^b
ET Sexed Sperm	40	41%	37%	38%

a,b Means within columns differ, $P < 0.05$.

Abortion and Calving Data

Overall abortion rates at each dairy were different (Dairy 1, 31% and Dairy 2, 15%);($P < 0.02$);(Table 5). For Dairy 1(Bulls A, B, C), there were 17 abortions between the 35 and 60 d pregnancy checks (17/104) (16% abortion rate of those pregnant) and 13 additional abortions between the 60 d pregnancy check and parturition (13/81);(16% of those pregnant excluding 17 aborted fetuses before day 60 and 6 cows that died after the 60 d pregnancy check);(Tables 4 and 5). The abortion rate was 79% for the X-sorted ET pregnancies at Dairy 1 (11/14) and 42% for X-sorted ET pregnancies for both dairies (13/31). Both were significantly higher than any other treatment group ($P < 0.01$), but the overall rate was high due to Dairy 1's results (Table 5). Bull A-1 pregnancies had a higher abortion rate than those of bull B-1 at Dairy 1 ($P < 0.02$), but abortion rates for those bulls (bull A-2 and bull B-2) were both low at Dairy 2, (Table 4).

Dairy 2 did not follow the same pattern as Dairy 1, although, early term abortions overall were similar for Dairy 1 and Dairy 2 ($P > 0.1$). Dairy 2 had only 1 late term abortion (1/59, 2%), which was significantly less than Dairy 2's late term abortion rate (13/81, 16%);($P < 0.01$). There were no stillborn calves recorded for Dairy 2.

Table 4. Early (between d 32 and 60) and Late (after d 60 to parturition) Embryonic and Fetal Death of Pregnancies (EDP) by Bull and Treatment (Control-C and Sexed-X) at Dairy 1 (top) and Dairy 2 (bottom).

Treatment	EDP	Bull A-1	EDP	Bull B-1	EDP	Bull C-1	Overall	
Early AI C	2/11	18%	0/18	0%	1/13	8%	3/42	7% ^f
Late AI C	1/9	11%	2/16	13%	2/12	17%	5/37	14% ^f
Early AI X	3/13	23%	1/13	9%	0/6	0%	4/32	12% ^f
Late AI X	3/9	33%	1/10	10%	0/6	0%	4/25	16% ^e
Early ET C	0/4	0%	0/8	0%	1/4	25%	1/16	6% ^f
Late ET C	1/4	25%	0/7	0%	1/3	33%	2/14	14% ^e
Early ET X	5/6	83%	1/5	20%	3/3	100%	9/14	64% ^d
Late ET X	0/1	0%	2/4	50%	-	-	2/5	40%
Overall	15/34	44%^a	7/44	16%^b	8/26	26%	30/104	23%

Treatment	EDP	Bull A-2	EDP	Bull B-2	Overall	
Early AI C	0/11	0%	1/9	11%	1/20	5%
Late AI C	0/11	0%	0/7	0%	0/18	0%
Early AI X	1/10	10%	3/15	20%	4/25	16%
Late AI X	0/9	0%	1/11	9%	1/20	5%
Early ET C	2/6	33%	0/4	0%	2/10	20%
Late ET C	0/3	0%	0/3	0%	0/6	0%
Early ET X	2/9	22%	0/8	0%	2/17	12%
Late ET X	0/7	0%	0/8	0%	0/15	0%
Overall	5/36	14%^c	5/36	14%^c	10/72	14%

a, b P < 0.02; a, c and d, e P < 0.01; d, f;

Means with different superscripts differ, P < 0.001, χ^2

N changes from early to late due to death of cows.

Table 5. Total Embryonic Death per Treatment for Dairies 1 and 2.

Treatment	Dairy 1 abortions	Dairy 2 abortions	Total abortions	Total percent aborted
AI Control	8/40* ^c	1/19* ^e	9/59*	15% ^a
AI X-sort	8/29* ^c	5/24* ^e	13/53*	24%
ET Control	3/15* ^c	2/8*	5/23*	22%
ET X-sort	11/14 ^d	2/17 ^e	13/31	42% ^b
Total	30/98* (31% ^f)	10/68* (15% ^g)		

a, b and c, d P < 0.01; e, f P < 0.001; g P < 0.02, Means with different superscripts differ, χ^2
 *Before parturition, 6 of the 104 cows died at Dairy 1, and 4 of 72 cows died at Dairy 2.

Included in calving data for Dairy 1 were 5 stillborn calves scattered among the treatment groups. Sex ratios of bulls to heifers for the four treatment groups (Table 6) were AI control sperm (26/50, 52%), AI sexed sperm (5/40, 12%), ET control sperm (9/18, 50%) and ET sexed sperm (2/18 11%).

Table 6. Sex ratio of calves at both dairies by treatment.

Treatment	Bull Calves	Percent Bulls	Heifer Calves	Percent Heifers
AI Control	26	52%	24	48%
AI Sexed	5	12%	35	88%
ET Control	9	50%	9	50%
ET Sexed	2	11%	16	89%

Discussion

IVP Embryos

Fertility of X-sorted sperm with AI was lower than that of unsorted sperm. This was similar to findings of others (Seidel and Garner, 2002; De Jarnette et al., 2010). IVP of embryos via IVF with sexed sperm was similar to previous findings (Cran et al., 1994; Lu et al., 1999; Wheeler et al., 2006) in that cleavage rates were generally similar between sexed and non-sexed sperm. However, the sperm concentration used was 4-fold higher for sexed than for control sperm. Despite similar cleavage rates, sexed sperm resulted in lower blastocyst production rates than control sperm, with individual bull effects. One bull (bull C) had fertile control sperm but a significantly lower cleavage rate with sexed sperm. The blastocyst rate per oocyte of this bull was not significantly lower ($P > 0.1$) than that of the bulls with a higher sexed sperm cleavage rate. This difference may be significant with additional replication. A difference in development of embryos produced with sexed sperm likely occurs via a delayed first cell cycle (Lu et al., 1999; Wheeler et al., 2006). Perhaps embryos produced with sexed sperm require specially tailored culture conditions. One example of embryo sex specificity was the discovery that replacement of glucose with fructose yielded more blastocysts; fructose was less harmful to female embryos than glucose (Barceló-Fimbres, 2007).

Use of sexed sperm may require more tailored treatments. If some difference in blastocyst development is due to binding of Hoechst 33342 to DNA, it may be beneficial to look into means of removing the dye before fertilization, although this would require

further manipulation of sperm that may already be partially capacitated (Lu et al., 1999; Schenk et al., 1999; Wheeler et al., 2006; Alomar et al., 2008).

Though time between semen collection and sperm sorting must be maximized for sexed sperm to be feasible, a more important consideration may be sorting pressure while sexing sperm (Lu et al., 1999; Seidel and Schenk, 2008). Additionally, because of great variation, bulls should be screened for in vitro fertilization success before commercial use. As with cleavage rates with IVF, Bull C had a significantly lower AI pregnancy rates using sexed sperm than control sperm and lower sexed AI pregnancy rates than the other bulls (Figures 1 and 4.). Bull fertility differences have been a common finding in studies that involve IVF (Alomar, 2006; Hansen, 2006) and sexed sperm (Wheeler, 2006), but the reason is poorly understood (DeJarnette et al., 2010). Alternative procedures for processing sperm may be necessary, for example altering concentrations of capacitating agents to optimize a particular bull's sperm fertility (Lu and Seidel, 2004; Alomar et al., 2008).

The incidence of polyspermy was not examined in this experiment, but was not found to be a contributing factor to bull infertility in another study (Alomar et al., 2006). Bulls may be differentially sensitive to capacitating agents, and it may be useful to study this in the future (Alomar et al., 2008; Garner, 2006; Maxwell et al., 2007; Schenk et al., 2008) to fully understand what components of sperm biology provide greater sperm longevity. In addition, other methods of treating sperm such as the addition of cell membrane stabilizing proteins help maintain acrosome integrity (Maxwell et al., 2007).

Technician and Dairy Differences

There were no significant differences in technician success rates, and the same technicians were used at both dairies. Cows from Dairy 1 may have been under more stress or had poorer nutrition. Both dairies had similar fertility rates when control and sexed sperm were used for AI. ET pregnancy results with embryos produced using sexed sperm were comparable to AI at one dairy but not the other. More ET numbers are needed to strengthen this observation. Others noted a strong correlation between farm management and cow fertility (Stroud and Hasler, 2006), which may have impacted results in this experiment.

Pregnancy Rates

Overall day 60 AI pregnancy rates for sexed sperm were 76% of control sperm ($P > 0.1$), which is similar to findings of others (DeJarnette et al., 2010) and make sexed sperm a promising choice if there is a need or market for a specific calf sex. However, one should expect fewer calves per AI with sexed sperm, especially with bulls unproven for sorted sperm. If a specific sex of calf is particularly valuable to a producer, slightly lower pregnancy rates may be justifiable. In addition, if oocytes harvested from a valuable cow are used for in vitro-produced embryos, it may be opportune to use sexed sperm. Pregnancy rates might have been higher from insemination later after onset of estrus than traditionally called for when using sexed sperm (Schenk et al., 2008). This would result in a shorter time from insemination to fertilization, which could assist sperm that has been sexed.

Overall Dairy 2 had fewer abortions and was managed in a way that caused less stress to each individual cow. Some of the management differences included individual sand bedding and calm handling at Dairy 2. Handling alone could cause significant stress to cows during scanning and treatment, which could cause a reduction of already poor fertility. Cows were also subjectively considered to be in better body condition on Dairy 2 than those on Dairy 1. Farm management differences attributing to body condition score can have a considerable impact on dairy cow fertility (Stroud and Hasler, 2006).

Abortion and Calving Data

There were unexpected cross bred calves from the embryo transfer group, two at Dairy 1 and six at Dairy 2, with one born dead. This was likely due to the quality control at the oocyte collection facility since all semen used was collected from the same 3 bulls, and AI groups did not have this problem. This makes finding a reliable supplier of oocytes for an ET program essential, if oocytes are not supplied by one's own cows.

Late term abortion rates for the first dairy were high. This is confounded by reproduction problems including abortions at that dairy that were occurring independent of the project. Dairy 2 had similar early abortion rates, but a significantly lower late term (after 60 d pregnant) abortion rate ($P < 0.01$). This could be attributed to differences in cow stress, nutrition, or health, but this study did not investigate these factors.

The calving sex ratio was similar to previous findings with heifer calves resulting in 88 and 89% of the calves for the X-sorted treatments and approximately a 1:1 ratio of sexes for untreated control groups.

Conclusions

Low cleavage rates of embryos produced in vitro with X-sorted sperm when compared to those of unsexed sperm may be an early indication of decreased viable pregnancies and early abortions for a particular bull. Bull C had significantly lower cleavage rates when using sexed sperm for IVF and had the lowest pregnancy rates for IVF embryos at 60 d. Early cleavage success or failure was not always reflected in blastocyst production and is still poorly understood. In this study, embryos produced in vitro using X-sorted sperm appeared to have consistently lower rates of blastocyst production than those produced with non-sorted sperm. However, studies tend to vary and may be related to the sires used. Likely due to fertility problems at the first dairy and low numbers for data analysis, no consistent correlation between stage of embryo and pregnancy rate was observed. Besides a seasonal difference, embryos at Dairy 1 were transferred 2 to 4 h later than those of Dairy 2. This may or may not have had a negative effect on embryos produced with sexed sperm. Although abortion rates were high at Dairy 1, especially with embryos produced with sexed sperm (79%); Dairy 2 had few abortions with this treatment (12%).

More data need to be collected to conclude individual benefits of applying certain technologies to a specific herd's reproduction program, but predictions can be made. In vitro embryo production is continually improving and is becoming competitive with AI in special situations (Hasler, 2006; Xu et al., 2009). IVP is an option for cows that have certain reproduction problems that may not be genetic in origin such as heat stress or an adhesion. Still, many aspects make IVP inferior from a practical standpoint. In vitro-produced embryos still lack the resilience of in vivo counterparts. Using sexed sperm

with superovulated cows may be more practical, but this was not explored in this experiment. Cryopreservation techniques for in vitro-produced embryos that are rapid, efficacious, and practical need to be perfected. Technical difficulty and expense of ET are greater than with traditional AI. In addition, further research is needed to determine what components of sperm affect fertility after being exposed to processing treatments.

Appendix1. Cryopreserved, In Vitro-Produced Embryos

The results for cryopreserved embryos were not included in the main body of the thesis because of poor results. There were 36 frozen thawed embryos transferred, and four pregnancies recorded. One of those pregnancies aborted early.

REFERENCES

- Al-Katanani Y.M., M. Drost, R.L. Monson, J.J. Rutledge, C.E. Krininger III, J. Block, W.W. Thatcher, and P.J. Hansen. 2002. Pregnancy rates following timed embryo transfer with fresh or vitrified in vitro produced embryos in lactating dairy cows under heat stress conditions. *Theriogenology*. 58: 171-82.
- Alomar M., J. Mahieu, B. Verhaeghe, L. Defoin and I. Donnay. 2006. Assessment of sperm quality parameters of six bulls showing different abilities to promote embryo development in vitro. *Reproduction, Fertility and Development*. 18: 395-402.
- Alomar M., H. Tasiauxa, S. Remaclea, F. Georgea, D. Paula and I. Donnay. 2008. Kinetics of fertilization and development, and sex ratio of bovine embryos produced using the semen of different bulls. *Animal Reproduction Science*. 107: 48-61.
- Barcelo-Fimbres M. 2007. Energy Substrates, metabolic regulators and lipid accumulation during the culture of in vitro produced bovine embryos. Ph. D. thesis, Colorado State University.
- Barcelo-Fimbres M., and G.E. Seidel, Jr. 2007. Effects of either glucose or fructose and metabolic regulators on bovine embryo development and lipid accumulation in vitro. *Molecular Reproduction and Development*. 74: 1406-18.
- Bermejo-Alvarez P., P. Lonergan, D. Rath, A. Gutierrez-Adan and D. Rizos. 2010. Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sexed sorted spermatozoa. *Reproduction Fertility and Development*. 22: 426-36.
- Cran D.G., D.J. Cochrane, L.A. Johnson, H. Wei, K.H. Lu, and C. Polge. 1994. Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF. *Theriogenology*. 41: 183 (Abstract)
- DeJarnette J.M., R.L. Nebel, and C.E. Marshall. 2010. Effects of 2.1 and 3.5 x 10⁶ sexed-sorted sperm dosages on conception rates of Holstein cows and heifers. *Journal of Dairy Science*. 93: 4079-85.
- De La Torre-Sanchez J.F., K. Preis, and G.E. Seidel, Jr. 2006a. Metabolic regulation of in-vitro-produced bovine embryos. I. Effects of metabolic regulators at different glucose concentrations with embryos produced by semen from different bulls. *Reproduction Fertility and Development*. 18: 585-96.

De La Torre-Sanchez J.F., D.K. Gardner, K. Preis, J. Gibbons, and G.E. Seidel, Jr. 2006b. Metabolic regulation of in-vitro-produced bovine embryos. II. Effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post compaction development on glucose metabolism and lipid accumulation. *Reproduction Fertility and Development*. 18: 597-607.

De Vries A., M. Overton, J. Fetrow, K. Leslie, S. Eicker and G. Rogers. 2008. Exploring the impact of sexed semen on the structure of the dairy industry. *Journal of Dairy Science*. 91: 847-56.

Drost M., J.D. Ambrose, M.J. Thatcher, C.K. Cantrell, K.E. Wolfsdorf, J.F. Hasler, and W.W. Thatcher. Conception rates after artificial insemination or embryo transfer in lactating dairy cows during summer in Florida. *Theriogenology*. 1999. 52: 1161-67.

Fernandez-Gonzalez R., P.N. Moreira, M. Perez-Crespo, M. Sanchez-Martin, M.A. Ramirez, E. Pericuesta, A. Bilbao, P. Bermejo-Alvarez, J. de Dios, F.R. de Fonseca, and A. Gutierrez-Adan. 2008. Long term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biology of Reproduction*. 78: 84-96.

Gardner D.K., M. Lane, and A.J. Watson. 2004. *A Laboratory Guide to the Mammalian Embryo*. Oxford University Press, Oxford, UK.

Garner D.L. 2001. Sex-sorting mammalian sperm: Concept to application in animals. *Journal of Andrology*. 22: 519-26.

Garner D.L. and G.E. Seidel, Jr., 2003. Past present and future perspectives on sexing sperm. *Canadian Journal of Animal Science*. 83: 375-84.

Garner D.L. 2006. Flow cytometric sexing of mammalian sperm. *Theriogenology*. 65: 943-57.

Garner D.L. and G.E. Seidel, Jr., 2008. History of commercializing sexed semen for cattle. *Theriogenology*. 69: 886-95.

Goichandran N. and Leese H.J. 2006. The effect of paracrine/autocrine interactions on the in vitro culture of bovine implantation embryos. *Reproduction*. 131: 269-77.

Hansen P.J. and Block J. 2004. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. *Reproduction, Fertility and Development*. 16: 1-14.

Hansen P.J. 2006. Realizing the promise of IVF in cattle- an overview. *Theriogenology*. 65: 119-25.

- Hasler J.F. 2003. The current status and future of commercial embryo transfer in cattle. *Animal Reproduction Science*. 79: 245-64.
- Hasler J.F. 2006. The Holstein cow in embryo transfer today as compared to 20 years ago. *Theriogenology*. 65: 4-16
- Heins B.J., L. B. Hansen, A.J. Seykora, A.R. Hazel, D.G. Johnson, and J.G. Linn. 2008. Crossbreds of Jersey x Holstein compared with pure Holsteins for body weight, body condition score, dry matter intake, and feed efficiency during the first one hundred fifty days of first lactation. *Journal of Dairy Science*. 91: 3716 - 22.
- Hosseini-Zadeh N.G., A. Nejati-Javaremi, S.R. Miraei-Ashtiani, and H. Kohram. 2009. Estimation of variance components and genetic trends for twinning rate in Holstein dairy cattle of Iran. *Journal of Dairy Science*. 92: 3411-21.
- Johnson L.A., J.P. Flook, and H.W. Hawk. 1989. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biology of Reproduction* 41; 199-203
- Lane M., D.K. Gardner, M.J. Hasler, and J.F. Hasler. 2003. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology*. 60: 407-19.
- Lazzari G., C. Wrenzyki, D. Herrmann, R. Duchi, T. Kruip, H Niemann, C. Galli. 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. *Biology of Reproduction*. 67: 767-75
- Loneragan P. 2007. State of the art embryo technologies in cattle. *Reproduction in Domestic Ruminants VI*. Nottingham University Press. Nottingham, UK. Pp. 315-25.
- Loneragan P. and T. Fair. 2008. In vitro produced bovine embryos- Dealing with the warts. *Theriogenology*. 69: 17-22.
- Lu K., D.G. Cran, and G.E. Seidel, Jr. 1999. In vitro fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology*. 52:1393-1405.
- Lucy M. C. 2001. Reproductive Loss in High-Producing Dairy Cattle: Where Will It End? *J.Dairy Sci*. 84:1277-93
- Lucy M.C. 2003. Mechanisms linking nutrition and reproduction in post partum cows. *Reproduction (Suppl)*. 61: 415-27.
- Lucy M.C. 2007. Fertility in high producing dairy cows: reasons for decline and corrective strategies for sustainable improvement. *Reproduction in Domestic Ruminants VI*. Nottingham University Press. Nottingham, UK. Pp. 237-54

Maxwell W.M.C., S.P de Graaf, R. El-Hajj Ghaoui and G. Evans. 2007. Seminal plasma effects on sperm handling and female fertility. *Reproduction in Domestic Ruminants VI*. Nottingham University Press. Nottingham, UK. Pp.13-38.

Nagy A., M. Gertsenstein, K. Vintersten, and R. Behringer. 2003. *Manipulating the Mouse Embryo; A Laboratory Manual*. 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, New York. Pp.161-76.

Olson S.E. and Seidel G.E. Jr. 2000. Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. *Biology of Reproduction*. 62: 248-52.

Panigone S., M. Hsieh, M. Fu, L. Persani, and M. Conti. 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. *Molecular Endocrinology*. 22: 924–36.

Park Y-S., S-S. Kim, J-M. Kim, H-D. Park, and M-D. Byun. 2005. The effects of duration of in vitro maturation of bovine oocytes on subsequent development, quality and transfer of embryos. *Theriogenology*. 64: 123-34.

Parrish J.J., J.L. Susko-Parrish, M.L. Leibfried-Rutledge, E.S. Critser, W.H. Eyestone and N.L. First 1989. Bovine in vitro fertilization with frozen–thawed semen. *Theriogenology* 25: 591–600.

Preis K.A., G.E. Seidel Jr. and D.K. Gardner. 2006. Reduced oxygen concentration improves the developmental competence of mouse oocytes following in vitro maturation. *Molecular Reproduction and Development*. 74:893-903.

Riddell K. and D. Stringfellow. 1998. The use of antibiotics in media for recovery, culture, and storage of embryos. In: D.A. Stringfellow, S.M. Seidel, eds. *Manual of the International Embryo Transfer Society*, 3rd ed. Savoy, IL: IETS; pp. 85-91.

Rizos D., A. Gutiérrez-Adán, S. Pérez-Garnelo, J. de la Fuente, M.P. Boland, and P. Lonergan. 2003. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction*. 68: 236–43

Shenk J.L. and Seidel G.E. 2007. Pregnancy rates in cattle with cryopreserved sexed spermatozoa: effects of laser intensity, staining conditions and catalase *Reproduction in Domestic Ruminants VI*. Nottingham University Press. Nottingham, UK. Pp. 165-77.

Schenk J.L., D.G. Cran, R.W. Everett, and G.E. Seidel Jr. 2009. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology*. 71: 717-28.

- Schiewe M.C. 1998. General hygiene and quality control practices in an embryo-production laboratory. In: D.A. Stringfellow, S.M. Seidel, eds. Manual of the International Embryo Transfer Society. 3rd ed. Savoy, IL: IETS; pp. 93-102.
- Seidel Jr. G.E., and L.A. Johnson. 1999. Sexing mammalian sperm-overview. *Theriogenology*. 52: 1267-72.
- Seidel, Jr. G.E., J.L. Schenk, L. Herickhoff, S.P. Doyle, Z. Brink, R.D. Green, and D.G. Cran. 1999. Insemination of heifers with sexed sperm. *Theriogenology*. 52: 1407-20.
- Seidel, Jr. G.E. and D.L. Garner. 2002. Current status of sexing mammalian spermatozoa. *Reproduction*. 124: 733-43.
- Seidel Jr. G.E., R.P. Elsdon, and J.F. Hasler. 2003. Embryo Transfer in Dairy Cattle. Hoard's Dairyman. Fort Atkinson, WI.
- Seidel Jr. G.E., and J.L. Schenk. 2008. Pregnancy rates in cattle with cryopreserved sexed sperm: Effects of sperm numbers per inseminate and site of deposition. *Animal Reproduction Science*. 105:129-38.
- Silva-del-Rio N., S. Stewart, P. Rapnicki, Y.M. Chang, and P.M. Fricke. 2007. An Observational Analysis of Twin Births, Calf Sex Ratio, and Calf Mortality in Holstein Dairy Cattle. *Journal of Dairy Science*. 90: 1255-64.
- Soto P., R.P. Natzke, and P.J. Hansen. 2003. Identification of possible mediators of embryonic mortality caused by mastitis: Actions of lipopolysaccharide, prostaglandin F_{2α}, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. *American Journal of Reproductive Immunology*. 50: 263-72.
- Stringfellow D.A. 1998. Recommendations for the sanitary handling of in-vivo-derived embryos. In: D.A. Stringfellow, S.M. Seidel, eds. Manual of the International Embryo Transfer Society. 3rd ed. Savoy, IL: IETS; pp. 79-84.
- Stroud B. and J.F. Hasler. 2006. Dissecting why superovulation and embryo transfer usually work on some farms but not others. *Theriogenology*. 65: 65-76.
- Takenaka M., T. Horiuchi, and R. Yanagimachi. 2007. Effects of light on development of mammalian zygotes. *PNAS*. 104:14289-93.
- Thibier M. 2009. Data Retrieval Committee statistics of Embryo Transfer- Year 2008. The worldwide statistics of embryo transfers in farm animals. *IETS Embryo Transfer Newsletter*. 27: 13-19.

- Touchberry R.W., 1992. Crossbreeding Effects in Dairy Cattle: The Illinois Experiment, 1949 to 1969. *Journal of Dairy Science*. 75: 640-667.
- Tubman L.M., Z. Brink, T.K. Suh, and G. E. Seidel, Jr. 2004. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. *Journal of Animal Science*. 82: 1029-1036
- Vajta G., T. Krosi, Y. Du, K. Nakata, S. Leda, M. Kuwayama, and Z. P. Nagy. 2008. The well of the well system: an efficient approach to improve embryo development. *Reproductive BioMedicine Online*. 17: 73-81
- Vasconcelos J.L.M., R.W. Silcox, G.J.M. Rosa, J.R. Pursley, and M.C. Wiltbank. 1999. Synchronilzation rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology*. 52:1067-1078.
- Wheeler M.B., J.J. Rutledge, A. Fischer-Brown, T. VanEtten, S. Malusky, and D.J. Beebe. 2006. Application of sexed semen technology to in vitro embryo production in cattle. *Theriogenology*. 65: 219-227.
- Willett E.L., W.G. Black, L.E. Casida, W.H. Stone, and P.J. Buckner. 1951. Successful transplantation of fertilized bovine ovum. *Science*. 113, 247.
- Wiltbank M., H. Lopez, R. Sartori, S. Sangritavong, and A. Gumen. 2006. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology*. 65:17-29.
- Wrathall A. E. and P. Suttmöller. 1998. Potential of embryo transfer control transmission of disease. In: Seidel SM, String fellow DA. eds. *Manual of the International Embryo Transfer Society*. 3rd ed. Savoy, IL: IET; pp. 17-44.
- Xu J., Z. Guo, L. Su, T.L. Nedambale, J. Zhang, J. Schenk, J.F. Moreno, A. Dinnyes, W. Ji, X.C. Tian, X. Yang, and F. Du. 2006. Developmental potential of vitrified Holstein cattle embryos fertilized in vitro with sex-sorted sperm. *Journal of Dairy Science*. 89: 2510-2518.
- Xu J., S.A. Chaubal, and F. Du. 2009. Optimizing IVF with sexed sperm in cattle. *Theriogenology*. 71: 39-47.
- Young L.E, K.D. Sinclair, and I Wilmot. 1998. Large offspring syndrome in cattle and sheep. *Reviews of Reproduction*. 3: 155-163
- Zhang M., K.H. Lu, and G.E. Seidel Jr. 2003. Development of bovine embryos after in vitro fertilization of oocytes with flow cytometrically sorted, stained and unsorted sperm from different bulls. *Theriogenology*. 60: 1657-1663.