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Sex Ratio of Embryos from Hyperstimluated Beef Cows

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SEX RATIO OF EMBRYOS FROM HYPERSTIMULATED BEEF COWS

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ABSTRACT

Sex selection is economically and genetically advantageous in cattle production. The only commercially available reliable sex selection technique is sexed semen; however, the technology is expensive and inefficient. Maternal manipulation could be beneficial to sway sex ratio. Frequent ultrasound coupled with rectal palpation has been shown to sway sex ratio towards female. In the preliminary study, cows with a corpus luteum (CL) ≥ 18 mm were randomly assigned to Treatment or Control (n=4 / group). Follicles > 5 mm were destroyed using ultrasound-guided ablation, a CIDR was inserted, and PGF2α (25 mg IM) was administered. Ovarian hyperstimulation began 48 h post-ablation and consisted of FSH administration (35 mg total IM) 2X/day for 4 days. Estrus detection began 24 h after CIDR removal (6^{th} FSH injection) and was conducted every 4 h until standing estrus. At estrus, GnRH (100 µg IM) was given and cows were inseminated with 2 doses of frozen-thawed semen from a single bull 12 h later. 24 h after CIDR removal, Treated cows were subjected to trans-rectal ultrasound evaluation of their reproductive tracts with a 7.5 mHz probe every 4 h for 40 h. Controls were processed through the chute, but not subjected to ultrasound. Day 7 embryos were collected via trans-cervical uterine lavage. Sex determination was conducted by combined duplex PCR and dot blotting. The number of ovulations and transferable embryos collected were similar between Treated and Controls (13.0±3.3 vs. 11.8±1.1; 5.8±2.3 vs. 6.2±3.7, respectively). The female embryo percentage did not differ between groups or in comparison the expected 50:50 ratio. Experiment 2 followed the same protocol except heifers were used (n=7/group), cattle were administered FSH reduced to 30 mg total dose

and control cattle were not subjected to chute processing. The number of ovulations and transferable embryos collected were similar between treated and controls (15.0±1.5 vs. 22.8±5.0; 5.7±2.2 vs. 6.8±2.5, respectively). The female embryo percentage did not differ between Treatment and Control; however, the Treatment and Control group combined (P=0.02) differed significantly from the expected value of 50:50.. Also, total embryos combined from the preliminary study and Experiment 2 differed from the expected value of 50:50 (P=0.02). This lead to Experiment 3 to determine if there was a timing of insemination effect. Cows were selected and hyperstimulated using the same protocol as the first two experiments, except FSH dosage was further reduced to 25 mg total dose and neither group was subjected to ultrasound. Cows (n=6/group) were randomly assigned to either 0 hour insemination group (inseminated at onset of estrus) or 12 hour insemination group (inseminated 12 hours after the onset of estrus). The number of ovulations did not differ from each other (9.5±2.0 vs. 7.7±1.1). There was a trend for the total number of transferable embryos to differ from one another (0.5±0.5 vs. 4.5±1.8). The female sex ratio could not be statically analyzed between each other because of the low frequency but did not differ when compared to the expected value of 50:50. Frequent ultrasound and rectal palpation around timing of insemination did not effectively skew sex ratio.

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CHAPTER ONE

INTRODUCTION

There are two available methods cattle producers can employ to obtain pregnancy within their herd. Natural service is the conventional method for breeding cows. In this management practice, a single bull or a few bulls contribute 50% percent of the genetics of the herd. This gives the bull a large role in the reproductive efficiencies of the herd placing a great deal of importance on bull fertility and breeding soundness coupled with the genetic merit of the bull. Other factors affecting bull viability are libido, mating ability, servicing capacity, and social dominance. The second method available to breed cows is artificial insemination (AI), an assisted reproductive technology.

Assisted reproductive technologies are used to meet reproductive, financial, and genetic goals within an operation. Artificial insemination is the most efficient and economical method for increased genetic performance of a herd using semen from genetically superior sires (Perry, 2005). Artificial insemination is an important tool available to cattle producers that will facilitate increased genetic gain, propagation of more elite genetics from outstanding bulls, rapid improvement of economic traits, increased efficiency of sire selection and progeny testing and a decreased number of bulls that need to be maintained on a farm (DeForest, 1983). With the use of AI, a superior bull may increase his progeny by more than a thousand fold, thus illustrating the importance of AI as a tool for genetic gain within a herd. With natural service, a single bull can service thirty to fifty cows per year for three to eight years yielding approximately four hundred calves in a lifetime, assuming a 100% conception rate, which is not realistic. With the technology of AI, this same bull can produce 200-400 units (straws) of semen per ejaculate at four ejaculates per week for 3-8 years. Assuming 1.5 units per calf this bull can sire

approximately five hundred thousand calves (Yarnell, 2004). Artificial insemination offers many other advantages, such as disease control, possibility of increased fertility, decreased cow injury due to natural service, and decreased cost associated with purchasing a bull and consequent management (Yarnell, 2004). From a management standpoint, for a cow to maintain a yearly calving interval, she must conceive again within 85 days post-calving (Clemson Extension, 2001). Artificial insemination implementation can reduce the amount of time necessary to breed cows, shorten the calving season and produce a more uniform calf crop at weaning providing the producer the added advantage of more pounds of calf to sell at weaning (Ishmael, 2001) and provide more predictable calving ease (Deforest, 1983; Johnson and Jones, 2003). Acceptable pregnancy rates using AI are dependent on several management factors such as proper nutrition before, during and after breeding, proper health status, accurate record keeping, organization and planning of the breeding program, estrous detection (if applicable), semen quality, storage and handling, adequate working facilities and skilled technicians (Clemson Extension, 2001). Several uncontrollable factors such as the weather, latitude and daylight can contribute to AI program efficiency as well (USDA, 2009; NAHMS, 2007-2008).

AI is currently used to breed 72.5% of dairy cattle, with timed AI (TAI) programs in 58.2% of dairy operations. This technology has played a crucial role in the marked 256% increase in fluid milk production per lactation seen over recent decades (Burns, 2000). In contrast, AI is implemented in only 7.6% of beef cattle in the United States (USDA, 2009; NAHMS, 2007-2008). There are many factors that contribute to the less frequent use of AI in beef cattle operations. Producers list labor, time and cost as the main reasons not to implement AI, followed by too difficult or complicated, lack of facilities and lower confidence in effectiveness (NAHMS, 2007-2008). Under the proper management strategy, AI can be used to

add value to a calf crop to sufficiently cover the added expense. The cost of A, compared to natural service, is estimated to be $5.55 more per pregnancy. This does not take into account the use of proven sires with above average genetics compared to risks associated with unproven bulls and lower quality genetics. A study at the University of Nebraska found that with an average bull cost of $1700, the price per pregnancy was the same for natural service versus AI (Ishmael, 2001). In another study using a model of $3000 for a bull, Etinger Cattle Company found that the cost of natural service was $20 greater per pregnancy than AI (Ishmael, 2001). In a well managed beef cattle operation, producers may be able to implement AI to realize higher economic and genetic gains.

Four important cornerstones of a successful AI program are cow fertility, the quality and handling of the semen, skill and experience of the AI technician, and perhaps the most crucial, excellent heat detection (Burns, 2000). Estrous synchronization can reduce the amount of time and labor needed for estrus detection and AI. The success of estrous synchronization programs relies heavily on proper estrous detection, because the detection efficiency correlates to pregnancy rates following AI or embryo transfer (ET). Synchronization programs call for estrus detection anywhere from three to eight days. This is labor intensive and in some cases cost prohibitive, as estrus detection costs range from $15-50 per cow per synchronization cycle (Gibbons, 2008). There are many factors that influence estrus detection efficiency, which directly affect the cost. These factors include: number of days needed for detection, the amount of time allowed for estrus detection per session and the frequency of detections per day. Other factors such as labor availability, facilities and management systems contribute to the ideal frequency and efficiency of detection. Fixed time AI is utilized in an ovulation synchronization program which eliminates the amount of labor needed for estrus detection because cattle are not examined

for estrus. This also ensures that every cow is inseminated, which adds the possible advantage of producing a pregnancy in a cow that might not have been observed in estrus with an estrus detection protocol, but does ovulate. AI, along with ovulation synchronization, facilitates the use of a timed AI program, which now achieve pregnancy rates comparable to those achieved by estrous detection programs.

Well managed operations are good candidates for AI and lend themselves to the application of sex ratio manipulation. Sex selection technology has been widely researched since the 1990's and is now commercially available. Using this technology, producers have the advantage of skewing sex ratios within their herds to utilize higher quality genetics and to take advantage of value differences in the price of male versus female progeny. Sexed semen is the only commercially available product that has been proven to effectively skew sex ratio. Current research focuses on finding alternative ways to alter sex ratio that are more economically advantageous in a commercial setting.

Application of Sex Selection Technology within the Dairy Industry

Within the dairy industry, there are two separate types of production systems. These two types of production systems are divided into commercial dairy production and seedstock dairy production, with each system unique in its target use for sex-selected progeny.

In a commercial dairy production system, a producer will breed a cow or heifer for two reasons. The first reason being to produce a pregnancy, that when carried through gestation and subsequent parturition, will cause lactation and fluid milk production. The second reason is to produce a replacement heifer or calf for sale.

In a study by Thatcher et al. (1980), a correlation was shown between the sex of a calf and the yield of lactation from that parturition. This study showed that cows carrying heavier

bull calves produced, on average, fourteen kilograms milk more at 305 day lactation than cows producing heifer calves. However, the slight increase in milk production would not counteract the increase in dystocia rates associated with the heavier bull calves (Sieber et al., 1989). Thus, the added advantage of sex selection towards male for increase in lactaction would be offset by an increase in calving difficulties.

When producing a calf for replacement or sale, sex determined progeny are more valuable to dairy producers. Producers could use sex selection technology on their higher quality females to increase the number of female calves for heifer replacement and male sex selection for lesser quality cows to gain added market value for calves not used for replacement stock (Hohenboken, 1999). Sex selection technology reduces the risk of disease from purchased replacement heifers and controls costs when generating replacements from within the herd (ABS Global, 2007).

To employ sex selection technology for the production of replacement heifers it would be necessary for dairy farmers to decide which cattle were of higher quality and which would be chosen to produce heifer calves to enter into their production systems. For these cattle, using sex selection technology to produce female calves would be of value and wouldn't be "wasting" the higher quality genetics of a superior cow on a bull calf, which are generally sold for veal or to be finished in a feedlot for considerably less than heifer calves. Using only the highest quality cows would increase selection pressure for production traits, creating even higher quality stock (Hohenboken, 1999).

After altering the sex ratio of the higher quality cattle's offspring toward female, three alternatives are presented for the insemination of the remainder of the cow herd. Studies by Van Vleck and Everett (1976), Van Vleck (1981), and Dematawewa and Berger (1998) proposed

inseminating the remaining cows and heifers with non-sexed dairy semen as a less expensive alternative. This low cost alternative would play a large role in offsetting the higher costs of using sexed semen (as a sex selection technology) on the higher quality cows of the herd. This would produce other heifer calves that could be retained or sold or bull calves that could be sold for commercial use (Hohenboken, 1999). Also, for value added production, producers could choose to breed the remainder of the herd with non-sexed beef semen. The crossbred progeny would result in higher market values for feeder calves with beef influence. However, in a study by Guilbault et al. (1990), a reduction in milk yield was found when crossing beef studs on dairy cows and heifers. To further add value to these crossbred calves, a producer could choose to inseminate the cows with male-sorted semen to add to the economic gain associated with beef breeds over dairy breeds for meat production, by coupling that with the added value of a steer over a heifer calf. Increased value of a steer calf over a heifer calf is directly influenced by a realized increase in feed efficiency and gain and an increase in carcass quality. However, an increase in dystocia rates is associated with an increase in male calves, especially within a younger cow population (Hohenboken, 1999).

Sex selection is valuable to seedstock dairy production if it can increase the rate of response to selection and make genetic change less expensive. For genetic improvement, a genetically superior bull is mated with genetically superior females to produce male progeny which are then bred to females to produce females for testing. Sex selection would be of great value to reduce the number of matings necessary to produce these crosses. In the initial cross of genetically superior cattle, male progeny are more valuable. If sexed semen is used as a sex selection technology, it would increase the possibility of a male by a factor of the probability of a male calf using sexed semen divided by the probability of a male calf using non-sexed semen,

.9/.2=4.5 for example. Therefore a mating using sexed semen would be four and a half times more likely to produce a male calf than a mating using non-sexed semen. An increase in selection intensity will also be realized as fewer, more elite dams are used (Van Vleck, 1981).

In the second matings necessary to produce daughters for progeny testing, daughters are more useful. Using non-sexed semen takes an average of six inseminations to produce one daughter who will complete a lactation cycle (Van Vleck and Everett, 1976). The use of sexed semen could lower the number of inseminations needed, which would lower the cost to producers allowing for more bulls to be progeny tested (Hohenboken, 1999).

Application of Sex Selection Technology within the Beef Industry

As with the dairy industry, the beef industry has two different production systems which would have different uses for sex selection technology. A commercial producer will breed a cow to produce calves destined for slaughter and calves for replacement heifers. Sexed semen can be used to designate maternal characteristics for replacement heifers and carcass traits for slaughter progeny. Using X-sexed semen a producer can select his more valuable cows to produce replacement heifers, increasing selection pressures, and in theory, creating successively superior genetic stock (Hohenboken, 1999).

Sexed semen could also be used to breed replacement heifer calves to produce heifer replacements. This shortens the genetic interval and as an added bonus lowers dystocia rates (Peck, 2000). Bull calves generally weigh 1.5-10 pounds more at birth than heifer calves and need 10-40% more assistance at birth. Because of physical immaturity, many heifers experience increased complications at calving. Dystocia results in a longer interval between calving, correlating to more days open, lowered pregnancy rates, lowered milk production, an increase in the incidence of retained placentas, cow deaths, and costs associated with veterinary care.

Difficulty calving reduces profits and costs consumers in the long term. Utilizing sexed semen could decrease dystocia rates by heifers being bred to produce heifer calves. These sexed pregnancies of replacement heifers could add $50-$100 to the value above that already paid for replacement heifers (Schenk and Seidel, 1999). By breeding heifers to carry heifer calves to reduce dystocia, producers would not have to sacrifice genetics by being limited to selecting low birth weight bulls (Lents et al., 2003).

Commercial beef producers could also employ Y-sexed semen to take advantage of the higher value of bull calves destined for market. Bull calves exceed heifer calves for traits such as growth rate, feed efficiency and conversion, muscling, fat deposition and carcass merits which meet the demands of the market. Here a producer could use sexed semen from a terminal sire to add value to the bull calves (Hohenboken, 1999).

A unique approach to commercial beef production facilitated by sexed semen is applying the technology to a single sex bred heifer production system (SSBH). Using SSBH, a producer can use heifer calves to produce replacements and then slaughter these animals. In the United States, cows less than 24 months of age will not take a break in price for maturity. Therefore, if a producer could breed heifers to produce a single pregnancy of a heifer calf, genetic replacement could take place within the two year window so these cows could be culled before they would take a price reduction in market value. This would also make cow production more efficient because a cow would be slaughtered at the appropriate time to take advantage of the largest portion of nutrient intake for muscle development as opposed to using a larger portion for reproduction. This single sex bred heifer production system was proposed by Taylor et al. (1985).

The applications of sexed semen within the seedstock beef industry are very similar to the applications within the seedstock dairy industry. Sexed semen is a valuable tool to increase selection pressures and decrease genetic interval, resulting in faster genetic process (Hohenboken, 1999).

Gender selection for offspring in a cattle production setting is genetically and economically valuable. Currently, sexed semen is the only commercially available reliable method to skew sex ratio. This technology is expensive and inefficient. Focus on the maternal component of gender selection could be advantageous. The aim of the research in the following thesis was to determine if ultrasound and rectal palpation around timing of insemination would skew sex ratio towards female in hyperstimulated cattle.

CHAPTER TWO

LITERATURE REVIEW

Preference, performance and value differences between male and female offspring within the beef and dairy industries have led to research focusing on skewing sex ratio. To date, the only commercially available, reliable sex altering technology is sexed semen. Currently, there are no inexpensive, effective methods for skewing sex ratio.

Sex Determination

Female haploid oocytes contain an X-chromosome and male haploid sperm cells contain either an X or a Y bearing chromosome. Thus, the sperm cell determines the sex of the resulting embryo because it contributes either an X or a Y chromosome. An XX genotype will produce a female offspring and an XY genotype will produce a male offspring (Kocer, 2009). It is believed that males produce X and Y bearing sperm in an equal ratio, resulting in equal male to female offspring ratios.

The SRY gene located on the short arm of the Y chromosome, which was discovered in 1990, is the sex determining region (Sinclair et al., 1990). If the SRY gene is present (XY genotype), the fetal gonads will differentiate into testis which develop Sertoli and Leydig cells that will subsequently produce anti-Mullerian Hormone (AMH) and masculinize the resulting embryo (Koopman et al., 1991). In cattle, the SRY gene can be detected as early as the four to eight cell embryonic stage by RT-PCR (Gutiérrez-Adán et al., 1997).

Techniques Available to Alter Sex Ratio

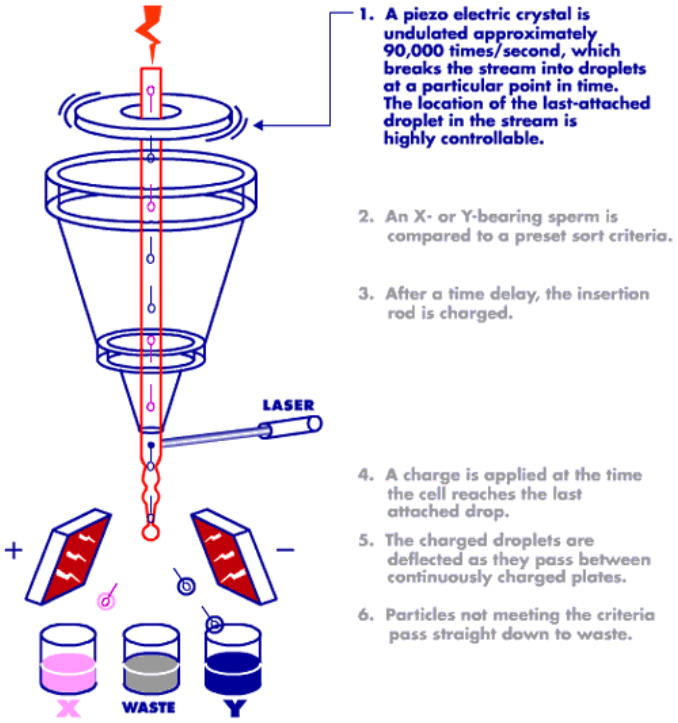
Flow Cytometry

The most common procedure used to sex and sort semen is DNA staining coupled with the use of a flow cytometer/cell sorter. This process resulted in the first sexed progeny in the 1990's. Developed by USDA researchers, the patent for this process for semen sexing and sorting was then sold to XY, Inc. in Fort Collins, Colorado, and has continued to be on the forefront of semen sexing technologies of non-human species (Peck, 2000). Flow cytometry can be used to sort cells based on cell mass. X bearing sperm cells contain 3.7-4.1% more DNA than Y bearing sperm cells (Garner et al., 1983). The difference in mass of the two different sperm cells makes it possible to use flow cytometry to sort these cells by mass.

To determine if a sperm cell contains an X or a Y chromosome, a sperm sample is diluted to a small concentration and stained with Hoeschst 33342, a dye that binds specifically to DNA and glows when exposed to ultraviolet light at approximately 360 nanometers wavelength. The X chromosomes have more DNA, and therefore bind to more Hoeschst 33342 and fluoresce brighter when excited. Once dyed, the sample is sent through the flow cytometer at 60 mph under 40-60 psi (pounds per square inch of pressure). A vibrating crystal breaks the flow into individual droplets, of which only 30% contain sperm. The sperm droplets pass through a laser which excites the dye and a detector reads the amount of fluorescence and transmits the reading into a computer program. A negative or positive charge is then assigned to each droplet, negative charges are assigned to Y bearing sperm cells, and positive charges are assigned to X bearing sperm cells. The droplets then pass through high voltage deflection plates which split the single stream into three separate streams. The negatively charged plate attracts the positively charged X-bearing sperm and positively charged plates attract the negatively charged Y-bearing sperm.

Uncharged droplets which contain either multiple sperm cells or unsexed sperm cells pass through a waste catcher in the center of the machine (DeForest, 1983, Schenck and Seidel, 1999, Deutscher et al. 2002, Seidel. 2002). Figure 1 details the technique of the flow cytometer.

Figure 2.1: Diagram of MoFlow Flow Cytometer



Source: www.xyinc.com

There are many factors which affect the effectiveness of the cell sorter, including sort speed and accuracy selection, which are relative in terms of speed versus accuracy. Generally, sperm is sorted at a rate of 3,000-4,000 sperm of each sex per second at 90% accuracy. At this rate, discounting post-sorting process losses, 10 million sperm are produced per hour or approximately the number of sperm contained in a single normal dose of unsexed sperm. To make the sorting process more efficient, lower numbers of sperm are contained in a sexed semen dose, usually around 2 million sperm cells, or 1/5 the sperm of a normal dose (Seidel, 2003). Also, the sort accuracy is affected by sperm morphology. Because of the rounded shape of the

head on the sperm, the laser must be exactly positioned to pass through the sperm head at the precise location on the flow cytometer to cause a useable reading. If the laser is deflected because of a wrong angle, the sperm is uncharged and discarded in the waste container (Schenk and Seidel, 1999). Post sort losses average 20% loss in sperm count due to damaged sperm, misreading, or unidentified sex (Seidel, 2003). Semen sorting tolerance and resulting fertility can also differ from bull to bull.

There are also several factors affecting the economic feasibility of this technology. MoFlow cell sorters developed by XY, Inc. sell for $300,000. Dr. Seidel of Colorado State University suggests that a rough estimate for the cost to start a lab which sorts semen will be approximately $2 million dollars for purchase and installation of two cell sorters, technicians, and operating costs for a single year (Seidel, 2003). In translation of that cost to the producer, a straw of sexed semen from a dairy bull ranges from $50 to $200, and varies significantly for beef bulls (Hansen, 2006). Although proven effective, flow cytometry generally requires a fresh semen sample and yields a reduction in integrity of the remaining sperm cells. Several questions have arisen from the use of the bis-benmididazole dye, Hoeschst 33342, as well. This dye targets the DNA at adenine-thymine rich regions (Yasui et al., 2007) and could potentially cause chromosomal abnormalities (Libbus et al., 1987). Sexed semen remains the only commercially available reliable sex selection technology.

Surface Antigens

Eichwald and Silmser (1955) discovered cell surface antigen differences while studying skin grafts in mice. These surface proteins have been suggested to differ between X and Y bearing sperm cells and binding of these different proteins have been thought to potentially aid in separation of the two types of sperm cells. Conflicting findings demonstrated only a fraction of

Y-bearing sperm cells expresses the H-Y antigen and antigen binding is variable (Sills et al., 1998). These shortcomings limit the application of sorting by surface antigen binding.

Albumin Separation

Albumin separation uses the progressive forward motility of sperm cells to separate Y bearing sperm cells from X bearing sperm cells (Ericsson et al., 1973). Ericsson et al. (1973) used the technique of washing and diluting semen prior to layering over columns of bovine serum albumin (BSA). They found that 85% of human Y sperm cells were isolated with 98% progressive forward motility. Using the same protocol, these results were not repeatable by Ross et al. (1975). Again the findings of Ericsson et al. (1973) were disputed, as Beal et al. (1984) using the same protocol but with bull semen as opposed to human semen, sorted the semen using flow cytometry after layering over the BSA column. They found no difference in percentage of X- and Y-bearing sperm cells in the Treatment (washed and diluted sperm) and Control (unaltered) groups. Conflicting results have limited the use of this technique.

Semen Incubation

Lechiniak et al. (2003) found that *in vitro* produced bovine embryos that were subjected to a 24 hour semen incubation period had a significantly higher proportion of hatched female blastocysts when compared to embryos subjected to a 0 or 6 hour semen incubation period. Another study found that when compared to X-bearing sperm cells, Y-bearing sperm cells had a lower percentage of motility, slower progression, slower velocities, and decreased hyperactivation after 24 hours of incubation (Watkins, 1996). No morphological differences were examined between the X and Y bearing sperm cells, so incubation effect and subsequent mechanism are not known.

Commercially Available Products

A post-thaw semen treatment is commercially available from Emlab Genetics (Arcola, Illinois), which claims to alter motility of the unwanted sex. HeiferPlus™ and BullPlus™ are two bovine semen treatment products said to alter the sex ratio towards female and male offspring, respectively. This treatment claims to stimulate motility in the sperm cells bearing the chromosome of the selected sex. Simultaneously, the motility of the sperm containing the undesired sex chromosome is stunted. At insemination, the increased motility of the selected sperm containing the desired sex will reach the ovum first. In theory, this results in more pregnancies of the desired sex. This is a patented technology so the mechanism is unknown. Emlab-sponsored research published on their website shows a significant skew in sex ratio in hyperstimulated and single ovulating cattle (Williams, 2007). A contrasting study by Curry et al. (2007) studied the ability of HeiferPlus™ treated and incubated semen to skew sex ratio as compared to simply incubated semen. Results concluded that the HeiferPlus™ did not successfully skew sex ratio towards female in the resulting offspring in either hyperstimulated nor single ovulating cattle. As with other semen altering technologies, there was significant bull to bull variation and timing of insemination could have muted the motility alteration.

Timing of Insemination

Insemination at differing intervals in relation to ovulation is another possible method to attempt to skew the sex ratio of the resulting offspring. Martinez et al. (2004) collected data from 716 cows inseminated at different intervals from the onset of estrus. The authors grouped all inseminations into three categories based on the interval from standing estrus to insemination (8-18, 18-30 and ≥ 30h). Their results showed a significant increase in the percentage of female calves (73.05%) produced from the early inseminations group. They also found that the

percentage females dropped at a rate of 1.85% per hour for each hour longer in the interval from estrus to breeding. The last insemination group produced a significantly higher percentage (72.06%) of male calves; however, a sharp decline in conception rates was also found in this insemination group.

Pursley et al., (1998) designed study to determine the optimum time to inseminate relative to the last gonadotropin releasing hormone (GnRH) injection in the synchronization protocol OvSynch. They found that cows inseminated at 0 and 32 hours following the GnRH injection produced the highest percentages of female calves.

In a recent study (Curry et al., 2009), Angus and Angus-cross hyperstimulated beef cows (n=25) were subjected to ultrasound-guided follicular ablation, administration of 25 mg PGF2α, and CIDR insertion. Hyperstimulation with 20 mg total dose of FSH began 48 h post ablation and continued with a decreasing dose over 4 d. On Day 3 of FSH administration, two doses of 25 mg PGF2α were given ~ 8 h apart. Cattle were examined for signs of estrus every 12 h and administered 100 µg of GnRH at estrus. Cattle were inseminated with either frozen-thawed semen that had been incubated for 20 min at 37° C or frozen-thawed semen that had been treated with HeiferPlus™ (Williams, 2007) and incubated at 37°C for 20 min. The HeiferPlus™ treated semen did not effectively sway the sex ratio; however, the Control group inseminated with strictly incubated semen produced a significantly ($P<0.005$) higher proportion of female embryos (106/265, 65%). These data suggest that timing of insemination is important, and with this synchronization protocol, effectively altered the sex ratio in favor of the female. In a hyperstimulation study with beef cattle, similar results were achieved (Davis et al., 2009). In the first experiment (Angus and Angus-cross cows; n=8) and the second experiment (Angus and Angus-cross heifers; n= 14) cattle were synchronized and hyperstimulated under the same

protocol and examined for estrus 36 h after the initial injection of PGF2α and every 4 h thereafter until estrus was observed in all animals. Cattle were given 100 µg GnRH at estrus and inseminated with two doses of frozen-thawed semen from a single ejaculate of a single bull 12 h later. The Treatment groups for these experiments were subjected to trans-rectal ultrasound every 4 h from 36 to 76 h after the initial PGF2α injection. No difference in sex ratio was detected between groups; however, in Experiments 1 and 2, the Control groups produced 56% and 71% female embryos, respectively, which when combined, differed significantly (P= 0.017) from the expected ratio of 50:50. When combining the Treatment (63%) and Control (66%) groups for both experiments, the ratio of female to male embryos was significantly (P=0.018) skewed towards the female when compared to the expected ratio of 50:50.

Several conflicting studies have found that altering the timing of insemination has no effect on sex ratio of subsequent offspring. Rorie et al. (1999) found that there was no difference in the sex of calves produced from insemination 10 and 20 hours prior to expected timing of ovulation. Also, Soede et al. (2000) showed that there was no difference in offspring sex from hogs that were inseminated at four hour intervals from the beginning of estrus until ovulation. Ideta et al. (2008) also found that altering timing of insemination did not affect the subsequent sex of the produced offspring. Conflicting results have limited the commercial application of altering the timing of insemination. However, variability of estrous detection and synchrony of time to ovulation may have produced varying results in these different studies.

Oviductal Proteins

Certain oviductal-specific proteins have been thought to potentially influence gamete and embryo development and viability by affecting either sperm capacitation or binding, fertilization and embryo development (Killian, 2007). Catt *et al.* (1997) found that *in vivo* produced ovine

embryos began with a higher percentage of male embryos, but at birth the ratios were again even suggesting that male embryos are less viable peri-implantation.

Cervical Mucosa

Wehner *et al*. (1997) conducted a study to test cervical mucosa impedance values in cattle and the relationship to the sex of resulting offspring. The impedance values were measured using the OVATECH intravaginal probe. They found that significantly more female calves were produces when impedance was declining and significantly more male calves were produced when impedance values were climbing.

Follicular Environment

Within the follicle, an elevated concentration of testosterone can possibly produce an oocyte with an apparent preference for fertilization by a Y-bearing sperm cell. Recently, a higher proportion of *in vitro* produced male embryos resulted from oocytes bathed in elevated follicular testosterone concentrations (Grant et al., 2008). This may suggest that there are differences in the fertilization capabilities of oocytes relative to the steroid environment, but not necessarily sex selection at the level of the sperm cell.

Maternal Stress

In a study by Pratt and Lisk (2004), female hamsters were stressed during early pregnancy (Days 5, 6 and 7). Treatment hamsters were placed with conspecifics and Control hamsters were introduced to novel areas. Within the Treatment group, one hamster was always dominant and the other subordinate. The subordinate females produced fewer male offspring than the dominant females. The authors suggested that the male embryo was more intolerant of uterine maternal stress. However, the dominant females could have potentially produced more testosterone which could have swayed sex ratio.

Environmental Factors

In some non-mammalian species, such as reptiles, sex is determined by temperature during incubation of the egg. This is an independent event not determined by specific sex chromosomes. Male and females both possess the same chromosomal characteristics. During a period of thermosensitivity, incubation temperature is thought to affect the undifferentiated gonads and induce differentiation into male or female dependent on the specific temperature (Pieu, 2004).

Temperatures at which different sexes develop can vary between reptilian species. Alligator embryos will differentiate into female at lower incubation temperatures and males at higher incubation temperatures. In contrast, turtle embryos will develop into male at lower incubation temperatures and higher incubation temperatures will result in female embryo development (Pieu, 2004). Because development into male or female embryos is dependent on incubation temperature, sex selection from a gamete manipulation standpoint has limited application to reptilian species.

Frequent Rectal Palpation/Ultrasound

In a study by Ideta et al. (2007), no difference in the sex ratio due to timing of insemination was found when hyperstimulated Holstein heifers were inseminated at either 48 or 56 h after PGF2α with frozen-thawed semen from multiple bulls (suggesting a possible bull effect). Hhowever, in a second experiment, they reported that heifers that produced a high percentage of female embryos (>50%) had a shorter duration of estrus (13.2±3.9 vs. 24.9±6.5 h), fewer standing mounts (27.8±5.9 vs. 75.0±18.5 mounts) and lower superovulatory response (8.4±1.0 vs. 19.7±3.1 ova collected) than heifers with a low percentage of female embryos (<50%). In the initial study (Ideta et al., 2008), they observed that heifers which had been

subjected to ultrasonic evaluation of their reproductive tracts to examine ovulation patterns tended to produce a higher number of female embryos (66.7%). These findings led to an additional study (Ideta et al., 2009) to determine the effects of frequent rectal palpation and ultrasonic evaluation on sex ratio. In this experiment, estrous synchronization was initiated by insertion of a progesterone releasing intra-vaginal device (PRID) for a period of 9 days. A PGF2α analog (Cloprosterol, 0.5mg) was administered 2 d before PRID removal and heifers were injected with equine chorionic gonadoptrophin (500 IU, IM) concurrently with PRID removal. Hyperstimulation treatment began mid-cycle (Days 8-10) with 8 decreasing doses of FSH (twice daily for 4 d, 28 Armour units total). PGF2α (dinoprost) was administered concurrently with the 7^{th} and 8^{th} FSH injections (25 mg and 15 mg, respectively). The Treatment group was rectally palpated and subjected to trans-rectal ultrasonography every 4 h from 36 to 76 h post initial PGF2α injection. All cattle were artificially inseminated 56 and 72 h post initial PGF2α with frozen-thawed semen from a single bull. Embryos were recovered on Day 7 after artificial insemination via uterine lavage. Sex of Grade 1-3 embryos was determined using loop-mediated isothermal amplification (Hirayama et al., 2004). In this study, the percentage of female embryos (Grades 1-3) was significantly ($P<0.05$) increased in the Treatment group (67.8%) from the expected ratio of 50:50. Blood was collected from the Treatment group every 4 h concurrently with ultrasonic evaluation. Blood was not collected from the Controls; however, blood was collected from three other hyperstimulated heifers every 12 h from 36 to 72 h post PGF2α as a cortisol control. In the frequent ultrasound group, the levels of blood cortisol were higher than the Controls. The peak cortisol level for the Treatment group was significantly ($P<0.05$) higher than the peak for the blood collection group. The authors proposed that acute stress around the time of ovulation skewed the sex ratio towards the female. Frequent rectal

palpation and ultrasound were used as the mechanism to deliver the acute stress (Ideta et al., 2009).

In two similarly designed studies (Davis et al., 2009), the authors found that frequent ultrasound around time of ovulation did not significantly skew the sex ratio toward the female. In Experiment 1, multiparous cattle were synchronized by ultrasound-guided follicular ablation, a CIDR was inserted and cattle were administered 25 mg PGF2α. Superovulation was initiated 48 h post-ablation with FSH (8 decreasing doses over 4 d, 35 mg total). Cattle were observed for estrus 36 h after initial PGF2α and every 4 h for 40 h and administered 100 μg GnRH at observed estrus. The cows were inseminated with two doses of frozen-thawed semen 12 h after estrus was observed. Cattle were then randomly divided into a Control and Treatment group (n=4/group). The Treatment group was subjected to trans-rectal ultrasound every 4 h beginning 36 h post initial PGF2α and continued for 40 h. The Control group was not subjected to ultrasound but was processed through the chute. Embryos were collected 7 d after insemination and graded based on the IETS scale (Stringfellow et al., 1999) and sex was determined on Grade 1-3 embryos using duplex PCR and Southern blotting. The percentage of female embryos (Grade 1-3) in the Treatment and Control groups were 57% and 56%, respectively. The sex ratio did not differ significantly from each other or from the expected 50:50 ratio.

In the second experiment by Davis *et al*. (2009), heifers were synchronized and hyperstimulated following the same protocol as described above. The Treatment group (n=8) was subjected to ultrasound every 4 h from 36 to 76 h post initial PGF2α and the Control group (n=7) was neither subjected to ultrasound nor processed through the chute. Day 7 embryos were collected and sex was determined on Grade 1-3 embryos using duplex PCR and Southern blotting. The percentage of females for the Treatment (palpation and ultrasound) and Control

(not subjected to ultrasound or chute processing) groups were 64% and 71%, respectively. These data suggest that the effect of the timing of insemination relative to the induced LH surge and subsequent ovulation (via GnRH) may have outweighed either the ultrasonic effect or the acute stressor effect within the experiment.

Because the only commercially available and reliable sex selection technology is expensive and inefficient, maternal side manipulation to skew sex ratio would be beneficial to the cattle industry. There are several potential options to skew sex ratio from the female component such as endocrinology manipulation, stress, age, parity and nutritional status of the donor cow and potentially, frequent ultrasound coupled with rectal palpation. The purpose of the following research was to determine if frequent ultrasound and rectal palpation around timing of insemination would skew sex ratio towards female in hyperstimulated beef cows and heifers. The hypothesis was that the frequent ultrasound and rectal palpation would skew sex ratio towards female and differ from the group not subjected to ultrasound or rectal palpation.

CHAPTER THREE

MATERIALS AND METHODS

Experiment 1

In Experiment 1, mature, multiparous Angus and Angus cross cattle were selected based on the presence of a corpus luteum (CL) with preference given to cattle with a CL greater than 18 mm. These cattle were then synchronized by ultrasound-guided follicular ablation of all follicles larger than 5 mm, a controlled internal drug release (CIDR; Eazi-Breed™ CIDR®, Pfizer Animal Health, New York, NY, USA) was inserted and cattle were administered 25 mg prostaglandin (PGF2α, dinoprost, 5 ml Lutalyse®, Pfizer Animal Health, New York, NY, USA). Superovulation was initiated 48 hours post-ablation with follicle stimulating hormone (FSH, Sioux Biochemical, Sioux Center, Iowa) in 8 decreasing doses for 4 days; each cow received 35 mg total FSH (Table 3.1).

Table 3.1: FSH Dosage for Hyperstimulation of Cows Subjected to Ultrasound (n=4) and Cows not Subjected to Ultrasound (n=4)

Day	AM Dose	PM Dose
Day 1	6.25 mg FSH	6.25 mg FSH
Day 2	5.00 mg FSH	5.00 mg FSH
Day 3	3.75 mg FSH 25.0 mg PGF2α	3.75 mg FSH 25.0 mg PGF2α Pull CIDR
Day 4	2.50 mg FSH	2.50 mg FSH

Estrus detection was aided by the use of a KAMAR® heat detection patch (Kamar, Inc., Steamboat Springs, CO) applied on the tailhead of each cow. Cattle were observed for estrus 36 hours after initial PGF2α for approximately 30 minutes every 4 hours until every animal was observed in estrus. They were administered 100 µg gonadorelin diacetate tetrahydrate (GnRH; Cystorelin®, Merial, Athens, GA, USA) at the onset of estrus. The cows were inseminated with

two doses of frozen-thawed semen from a single ejaculate of a single bull 12 hours after estrus was observed. Cattle were then randomly divided into a Control and Treatment group (n= 4/group). The Treatment group was subjected to trans-rectal ultrasound with a 7.5 MHz probe (Aloka, Tokyo, Japan) of their reproductive tract (ovaries and uterus) for approximately 2.5 minutes every 4 hours beginning 36 hours post initial PGF2α and continuing for 40 hours (10 sessions total). The Control group was not subjected to ultrasound but was processed through the chute.

Experiment 2

The purpose of Experiment 2 was to eliminate the possible additional stressor of chute processing in the Controls and determine the ultrasound effect around time of ovulation within the Treatment group. In Experiment 2, Angus and Angus cross heifers were selected based on the presence of a CL with preference given to cattle with a CL greater than 18mm. The heifers were synchronized by ultrasound-guided follicular ablation of all follicles greater than 5 mm, a CIDR (Eazi-Breed™ CIDR®, Pfizer Animal Health, New York, NY, USA) was inserted and cattle were administered 25 mg PGF2α (dinoprost, 5 ml Lutalyse®, Pfizer Animal Health, New York, NY, USA). Superovulation was initiated 48 hours post-ablation with FSH (Sioux Biochemical, Sioux Center, Iowa) for 8 decreasing doses over 4 days (30 mg total). Total FSH (Table 3.2) dose was stepped down from the first experiment to decrease the number of unfertilized oocytes collected and improve collection efficiency. Also, heifers were used in this experiment and they tend to be overstimulated by a dose a multiparous cow would receive.

Table 3.2: FSH Dosage for Hyperstimulation of Heifers Subjected to Ultrasound and Chute Processing (n=7) and Heifers not Subjected to Ultrasound or Chute Processing (n=7)		
Day	**AM Dose**	**PM Dose**
Day 1	6.25 mg FSH	5.00 mg FSH
Day 2	5.00 mg FSH	3.75 mg FSH
Day 3	3.75 mg FSH 25.0 mg PGF2α	2.50 mg FSH 25.0 mg PGF2α Pull CIDR
Day 4	2.5 mg FSH	1.25 mg FSH

Cattle were observed for estrus 36 hours after initial PGF2α and every 4 hours until all animals were observed in estrus and consequently administered 100µg GnRH (Cystorelin®, Merial, Athens, GA, USA) at the onset of estrus. The heifers were inseminated with two doses of frozen-thawed semen from a single ejaculate of a single bull 12 hours after estrus was observed. Cattle were then randomly divided into a Control and Treatment group (n = 7/group). The Treatment group was subjected to trans-rectal ultrasound every 4 hours beginning 36 hours post initial PGF2α and continuing for 40 hours (10 sessions total). The Control group was not subjected to ultrasound nor processed through the chute to eliminate possible addition of a chute processing stressor.

Experiment 3

The purpose of Experiment 3 was to determine if there was a timing of insemination effect on sex ratio by insemination at 0 or 12 hours after the onset of estrus. In Experiment 3, mature, multiparous Angus and Angus cross cattle were selected based on the presence of a CL with preference given to cattle with a CL greater than 18 mm. Cattle were synchronized by ultrasound-guided follicular ablation of any follicle larger than 10 mm, a CIDR (Eazi-Breed™ CIDR®, Pfizer Animal Health, New York, NY, USA) was inserted and cattle were administered 25 mg PGF2α (dinoprost, 5 ml Lutalyse®, Pfizer Animal Health, New York, NY, USA).

Superovulation was initiated 48 hours post-ablation with FSH (Sioux Biochemical, Sioux Center, Iowa) given in 8 decreasing doses over 4 days (25 mg total). The dosage of FSH (Table 3.3) was decreased from the previous experiments to further improve collection data and decrease the number of unfertile oocytes collected.

Table 3.3: FSH Dosage for Hyperstimulation of Cows Inseminated at 0 Hours after the Onset of Estrus (n=6) and Cows Inseminated at 12 Hours after the Onset of Estrus (n=6)

Day	AM Dose	PM Dose
Day 1	5.00 mg FSH	5.00 mg FSH
Day 2	3.75 mg FSH	3.75 mg FSH
Day 3	2.50 mg FSH 25.0 mg PGF2α	2.50 mg FSH 25.0 mg PGF2α Pull CIDR
Day 4	1.25 mg FSH	1.25 mg FSH

Cattle were observed for estrus 36 hours after initial PGF2α and every 4 hours for 40 hours. Cattle were randomly divided into a 0 hour (0 H) and a 12 hour (12 H) group (n = 6 / group). 0 H cows were inseminated with 2 doses of frozen-thawed semen from a single ejaculate of a single bull at 0 hour after observed estrus concurrent with administration of 100 µg GnRH (Cystorelin®, Merial, Athens, GA, USA). The 12 H group was given 100 µg GnRH (Cystorelin®, Merial, Athens, GA, USA) at the onset of estrus and inseminated with 2 doses of frozen-thawed semen from a single ejaculate of a single bull 12 hours later.

Embryo Collection

For all experiments, embryos were collected from Treatment and Control cattle on Day 7 post-insemination by trans-cervical uterine lavage using 500 ml of phosphate buffered saline (PBS) enriched with 10% fetal calf serum (FCS) and 10,000 units of penicillin / streptomycin. Embryos were located, rinsed, and evaluated (IETS grading system) microscopically. Embryos

were placed in 0.2 ml polymerase chain reaction (PCR) tubes with approximately 3µl of Emcare Embryo Holding Solution (Agtech, Inc., Manhattan, KS) and frozen at -20°C for future assay.

DNA Isolation

Frozen day 7 embryos were warmed to room temperature and 3 µl of Proteinase K (1 µg/µl; Sigma-Aldrich, St. Louis, Missouri, USA) was added. Tubes were placed in a thermocycler (Mastercycler® gradient; Eppendorf, Ontario, Canada) and incubated at 55° C for 12 min for degradation of the zona pellucida and lysis of cell membranes. Embryos were then heated to 98° C for 10 min to deactivate the Proteinase K. Embryos were held at 4° C for immediate use in PCR assay.

PCR

Primer sets were generated to the genomic bovine β-actin gene and bovine SRY (Daneau et al, 1995). The β-actin primer was 385 base pairs (forward: 5'-CCG AGG ACT TGA TTG TAC ATG G-3'; reverse 5'- ACT GGT CTC AAG TCA GTG TAC AGG-3'). The SRY specific primer set generated was 532 base pairs in length (forward: 5'-TCT TCC TTG TGC ACA GAC AG-3'; reverse: 5'-TTA TTG TGG CCC AGG CTT GT-3'). Primers were diluted to 250 pmoles/µl in Tris/EDTA (TE) stock and then diluted 1:10 in DepC water for a working stock. PCR mixture included:12.5 µl Go-taq (Promega), 1 µl of each β-actin primer and 2 µl of each SRY primer, template and sterile water were added for a final reaction volume of 25 µl. PCR was performed in an Eppendorf gradient thermocycler. Optimal reaction conditions were 95° C for 3 min followed by 35 cycles of denaturing at 94° C for 30 sec, primer annealing at 60° C for 30 sec, extension at 72° C for 30 sec, a hold at 72° C for 3 min and then a final hold at 4° C. PCR products were subjected to gel electrophoresis on a 1.6% agarose gel containing 4 µl ethidium bromide 1% (Fisher Scientific, Fair Lawn, New Jersey, USA) in 0.5X Tris/Borate/EDTA (TBE)

buffer at 100v for 30 min. Product was visualized by exposure to ultraviolet light. Presence of one band (at 385 bp, β-actin) indicated a female embryo and the presence of two bands (one at 385 bp, β-actin and another at 532 bp, SRY) indicated a male embryo.

Dot Blotting

PCR product was denatured with 0.1 vol 1N NaOH, incubated at 37°C for 5 min, then neutralized by the addition of 20X SSPE (3 M NaCL, 0.2 M NaH_2PO_4, 0.02 M EDTA) to a final concentration of 6X. Using a dot blot manifold, samples were added to 6X SSPE pre-soaked nylon membrane (Biobond™ - Plus Nylon Membrane; Sigma, St. Louis, Missouri, USA) over two pieces of Whatman paper. Embryo samples were run in duplicate, along with positive controls ranging from 2.0 ng to 0.08 ng of DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, Valencia, CA, USA). All membranes were cross-linked twice (Spectrolinker; Spectronics Corporation, Westbury, NY, USA) under UV light. Membrane hybridization overnight at 55°C was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK). Blots were washed and product detection was conducted by the addition of CDP-Star™ chemilumenescent detection reagent (GE Healthcare). The blots were exposed for 30 min using AlphaInnotec Imager. Membranes were first probed for the SRY gene segment and then stripped in boiling 6X SSPE/ 0.5% SDS for 15 min twice. Membranes were then probed for the β-actin segment.

CHAPTER FOUR

RESULTS

Number of ovulations, total number of ova collected, and the number of transferable embryos collected (collection parameters) were analyzed for differences between groups in each of the three experiments in this study. Data were analyzed using a student's t test in JMP (Version 8.2, SAS Institute, Cary, NC). Differences were reported at $\alpha \leq 0.05$ and a trend was recognized at $\alpha \leq 0.10$. A significant difference in any collection parameter would indicate a treatment effect on the efficiency of the collection, thus, differences were not expected. Experiments 1 and 2 showed no statistical difference in any parameter evaluated (Table 4.1-4.2). In Experiment 3, there was no significant difference between the 0 Hour and 12 Hour groups for number of ovulations recorded or total number of ova collected (Table 4.3). However, there was a trend (P= 0.06) in a reduction of transferable quality embryos collected from the 0 Hour group when compared to the 12 Hour group.

Table 4.1: Efficiency of Embryo Collection in Cows Treated with Ultrasound (n=4) and Non-treated Cows (n=4)

Collection Parameter	Treatment	Control	P-value
# of Ovulations	13.0 ± 3.3	11.8 ± 1.1	0.73
Total # of Ova	11.5 ± 5.8	10.0 ± 2.5	0.82
# of Transferrable Embryos	5.8 ± 2.3	6.2 ± 3.7	0.85

Table 4.2: Efficiency of Embryo Collection in Heifers Treated with Ultrasound (n=7) and Non-treated Cows (n=7)

Collection Parameter	Treatment	Control	P-value
# of Ovulations	15 ± 1.5	22.8 ± 5.0	0.14
Total # of Ova	11.9 ± 3.7	17.3 ± 3.5	0.30
# of Transferable Embryos	5.7 ± 2.2	6.8 ± 2.5	0.74

Table 4.3: Efficiency of Embryo Collection in Cows Inseminated at 0 Hours after Estrus Detection (n=6) or 12 Hours after Estrus Detection (n=6)			
Collection Parameter	**0 Hour**	**12 Hour**	**P-value**
# of Ovulations	9.5 ± 2.0	7.7 ± 1.1	0.44
Total # of Ova	7.8 ± 2.8	9.8 ± 3.5	0.66
# of Transferrable Embryos	0.5 ± 0.5	4.5 ± 1.8	0.06

Sex ratio for the Treatment and Control groups in Experiments 1 and 2 were analyzed using chi-square analysis (Preacher, 2001). Yates' correction for continuity was applied in situations where the frequency was below 5. This correction improves the accuracy for the null-conditioning sample distribution of the chi-square. Differences were reported at $\alpha \leq 0.05$ and a trend was recognized at $\alpha \leq 0.10$. In Experiment 1, sex was determined on 37 transferable-quality embryos, of which 21 (56.8%) were female. The Treatment group was (8/14) 57.1% female and the Control group was (13/23) 56.5% female. In Experiment 2, sex was determined on 82 transferable-quality embryos, of which 56 (68.3%) were female for the Treatment and Control groups combined. The female percentage of embryos in Experiment 2 were (26/40) 65.0% for the Treatment group and (30/42) 71.4% for the Control group.

Sex ratio was also evaluated within the Treatment and Control groups for both Experiment 1 and 2 by stage of the embryo. Stage 4 and 5 embryos were evaluated as earlier embryos and stages 6 and 7 were evaluated as older embryos. No significant differences in sex ratio were found between the groups for either Experiment 1 (Table 4.4) or 2 (Table 4.5), regardless of stage of embryo or treatment by subjection to ultrasonic evaluation of the reproductive tracts.

Table 4.4: Female Sex Ratio of Transferable Embryos in Cows Receiving Ultrasound Around Time of Ovulation and Processed Through the Chute (n=4) or Cows Only Processed Through the Chute (n=4)			
Endpoint	**Treatment**	**Control**	**Main Treatment Effect P-value**
Stage 4 and 5 Transferable Embryos	(4/6) 66.6%	(5/11) 45.5%	0.40
Stage 5 and 6 Transferable Embryos	(4/8) 50.0%	(8/12) 66.7%	0.46
Total Transferable Embryos	(8/14) 57.1%	(13/23) 56.5%	0.97

Table 4.5: Female Sex Ratio of Transferable Embryos in Heifers Receiving Ultrasound Around Time of Ovulation and Processed Through the Chute (n=7) or Heifers Who Were Neither Subjected to Ultrasound or the Chute Processing (n=7)			
Endpoint	**Treatment**	**Control**	**Main Treatment Effect P-value**
Stage 4 and 5 Transferable Embryos	(16/24) 66.7%	(22/31) 71.0%	0.73
Stage 5 and 6 Transferable Embryos	(10/16) 62.5%	(8/11) 72.7%	0.58
Total Transferable Embryos	(26/40) 65.0%	(30/42) 71.4%	0.53

Sex ratio was then compared between experiments with cows and heifer data were combined by treatment. Treatment and Control groups were evaluated between the experiments as well as total number of embryos from Experiments 1 and 2 were compared because there was no treatment effect between or within experiments. There was no difference between Treatment, Control, and Total Embryos between Experiments 1 and 2 (Table 4.6).

Table 4.6: Female Sex Ratio of Transferable-Quality Embryos in Cows (Exp. 1) or Heifers (Exp. 2) Subjected to Ultrasound or Not			
Endpoint	**Experiment 1 (Cows)**	**Experiment 2 (Heifers)**	**P-Value (Comparing Ex 1 vs Ex 2)**
Ultrasound Treatment Group Transferable Embryos	(8/14) 57.1%	(26/40) 65.0%	0.60
Non-Ultrasound Control Group Transferable Embryos	(13/23) 56.5%	(30/42) 71.4%	0.22
Total Transferable Embryos	(21/37) 56.8%	(56/82) 68.3%	0.22

Table 4.7 illustrates the sex ratio compared to the expected value of a 50:50 ratio. There were no differences when comparing the Treatment groups separate and combined to the expected value of 50:50 for Experiments 1 and 2. There was also no significant difference when comparing the Control groups of Experiment 1 and the Treatment and Control embryos combined for Experiment 1 to the expected value of 50:50. However, the Control groups of Experiments 1 and 2 combined, both Treatment and Control groups combined for Experiment 2 and both Treatment and Control groups combined for both Experiments 1 and 2 showed a significant difference ($p \leq 0.05$) when compared to the expected ratio of 50:50. There was also a trend for the Control group for Experiment 2 to differ from the 50:50 ratio. Because there was no ultrasound treatment effect and all embryos combined differed from the expected value of 50:50 this suggested that there was an alternate effect which skewed sex ratio towards female in all cattle. This suggests a possible timing of insemination effect (which has been shown to sway sex ratio) and potentially outweighed any other effects, such as ultrasound or stress.

Table 4.7: Female Sex Ratio Compared to Expected 50:50 Percentage Experiments 1 and 2 *1		
Endpoint	**Female Embryos**	**P-Value (Compared to 50:50)**
Experiment 1 Transferable Embryos-Treatment	(8/14) 57.1%	0.70
Experiment 2 Transferable Embryos-Treatment	(26/40) 65.0%	0.17
Experiment 1 and 2 Transferable Embryos-Treatment	(34/54) 63.0%	0.17
Experiment 1 Transferable Embryos-Control	(13/23) 56.5%	0.66
Experiment 2 Transferable Embryos-Control	(30/42) 71.4%	0.03*
Experiment 1 and 2 Transferable Embryos-Control	(43/65) 66.2%	0.06
Experiment 1 Total Transferable Embryos (Treatment and Control)	(21/37) 56.8%	0.56
Experiment 2 Total Transferable Embryos (Treatment and Control)	(56/82) 68.3%	0.02*
Experiment 1 and 2 Total Transferable Embryos (Treatment and Control)	(77/119) 64.7%	0.02*

1. Experiment 1 (Cows) and Experiment 2 (Heifers)

The purpose of Experiment 3 was to determine the effects of timing of insemination on sex ratio of embryos collected from hyperstimulated cattle. As previously mentioned, fertility was altered in the 0 Hour breeding group which has potential industry application as many commercial embryo collection protocols call for a breeding at 0 Hours and then subsequent breedings thereafter. In Experiment 3, sex was determined for 25 embryos, of which 13 (56.5%) were female. Chi-square analysis was not able to determine an appropriate P-value when comparing the 0 Hour and 12 Hour groups because the frequency was less than 1 in the 0 Hour

group, therefore no statistical relevance could be determined when comparing the two groups. The female sex ratio of the embryos collected for each group is reported in Table 4.8.

Table 4.8: Female Sex Ratio of Transferable Embryos in Cows Inseminated at 0 Hours after Estrus Detection (n=6) or Cows Inseminated 12 Hours after Estrus Detection (n=6)

Endpoint	0 Hour	12 Hour
Stage 4 and 5 Transferable Embryos	(0/2) 0%	(11/18) 61.1%
Stage 6 and 7 Transferable Embryos	(0/0) 0%	(3/5) 60.0%
Total Transferable Embryos	(0/2) 0%	(14/23) 60.9%

There were no differences in the female sex ratio for the appropriate groups as compared to the expected ratio of 50:50 for Experiment 3 (Table 4.9).

Table 4.9: Female Sex Ratio Compared to Expected 50:50 Ratio in Cows Inseminated at 0 Hours after Estrus Detection (n=6) or Cows Inseminated 12 Hours after Estrus Detection (n=6)

Endpoint	Female Embryos	P-Value (Compared to 50:50)
Experiment 3 Transferable Embryos- (12 Hour)	(14/23) 60.9%	0.46
Experiment 3 Transferable Embryos- (0 Hour and 12 Hour)	(14/25) 56.0%	0.67

CHAPTER FIVE

DISCUSSION

The rationale for this study was to investigate a method to skew the sex ratio towards more female offspring by manipulation of the dam. Focus was placed on manipulation from the female side because currently, the only commercially available option for sex ratio alteration in the cattle industry is sexed semen. This technique is expensive and inefficient. Other techniques are simply unreliable. Thus, a new focus on the dam side would be beneficial.

Ideta et al. (2007, 2009) found that frequent rectal palpation and ultrasound of the reproductive organs of a cow skewed the sex ratio of embryos collected from these cows towards female when compared to the expected value of a 50:50 (female:male) ratio. The authors of that study proposed that the delivery of stress by frequent rectal palpation was the cause of a larger proportion of female embryos collected. Pratt and Lisk (2004) support the theory that *in-utero* stress may alter sex ratio. They found that hamsters subjected to social stress (placed with an aggressive conspecific) produced less male offspring. In an attempt to emulate the Ideta *et al*. (2009) study, an experiment was designed to test the effects of ultrasound and frequent rectal palpation around timing of ovulation on sex ratio of embryos recovered from hyperstimulated beef cattle.

Ideta et al. (2007) found that timing of insemination had no effect on the sex ratio of embryos collected from hyperstimulated Holstein heifers which supports data from Rorie et al. (1999) and Soede et al. (2000). However, this contradicts findings of Martinez (2004), Pursley et al. (1998), Curry et al. (2009) and Davis et al. (2009) which did find a significant difference in sex ratio based on the timing of insemination. A timing of insemination effect could have potentially been masked in the Ideta et al. (2007) study because timing of insemination was not

relative to ovulation (in that study no GnRH was given) but to hours after the initial PGF2α injection. Cattle were also examined for estrus and FSH treatment was initiated Day 8 of the following estrous cycle. Also, the Treatment (frequent rectal palpation and ultrasound) and Control groups were not statistically analyzed against one another, only against the expected value of 50:50.

In our study, several changes were made from the Ideta et al. (2009) study to more accurately measure if ultrasound/frequent rectal palpation skewed sex ratio. Cattle were selected for the experiment based on the presence of a corpus luteum (CL) and cattle with a CL > 18 mm were preferred. Cattle were synchronized by follicular ablation of any follicle larger than 5 mm to tighten synchrony. Also, timing of insemination was more closely coordinated with the time of ovulation by checking for estrus every 4 hours and administering GnRH at the onset of estrus to standardize the interval from the onset of estrus to ovulation for each animal. Statistical comparisons were made between Treatment and Control groups as well as to the expected ratio of 50:50.

Experiment 1

No statistical differences were observed in the collection parameters (number of ovulations, total ova collected and number of transferable embryos collected) for this experiment. This indicated that the treatment had no effect on the collection efficiency and did not alter collection parameters. There was also no difference in the sex ratio between the Treatment and Control groups, or between the Treatment group and the expected ratio of 50:50. However, any effect in this experiment could have been masked by the low numbers (n=4/group). In retrospect, blood sampling at 4 hour intervals concurrent with rectal palpation and ultrasound could have potentially indicated whether or not the cattle were stressed by the

frequent capture, palpation and ultrasound by measuring cortisol levels in the blood. Also, in this study beef cattle of Angus and Angus cross decent were used; the previous studies by Ideta et al. (2007, 2009) examined the effects in Holstein heifers. There could be an inherent difference in the expected sex ratio between beef and dairy breeds because of differences in management styles and stress tolerance. A second experiment with larger numbers was needed to determine if any actual skewing of sex ratio can be attributed to the frequent rectal palpation and ultrasound and to eliminate chute stress as a potential factor in the Control group. It is important to note that there were many factors that could have swayed sex ratio towards female in the Treatment group in the Ideta et al. (2009) study, including the simple act of capturing the animal, the frequent rectal palpation and a direct effect of the ultrasound on the reproductive organs of the cow.

Experiment 2

A second study was performed imposing the same selection criteria; however, in the second study, only nulliparous heifers were used. The Control group was not subjected to chute processing and the Treatment group was frequently rectally palpated and subjected to ultrasound of their reproductive tracts. Also, to improve collection efficiency (decrease the number of unfertile oocytes collected) and because the experimental units were heifers, the FSH dosage was stepped down for this experiment (total dose decreased by 5 mg). No significant differences were reported in collection parameters, as expected, so treatment did not alter the collection efficiency. In this experiment, the Treatment and Control groups were not significantly different from each other when comparing sex ratio; however, the Control group (n=7) and the Treatment and Control groups (n=7 per group) together differed from the expected ratio of 50:50. This could potentially have been attributed to the decrease in the FSH dosage or impacted by the age of the donor (heifer vs. cow). Although, it is more likely that the larger number of heifers (n=14) in this

experiment more clearly depicted a timing of insemination effect. If stress is a factor, it is also a possibility that heifers are less accustomed to associated management practices and are thus more impacted by the stress of the procedures. Also, parity could potentially affect sex ratio and affect the differences in sex ratio between heifers and cows. A study by Huck *et al*. (1988) found that the percentage of male offspring increased with subsequent parities in hamsters.

When the embryos collected in Experiment 1 were pooled with the Experiment 2 embryos because there was no treatment effect, they differed statistically from the expected ratio of 50:50. Because all embryos combined differed from the expected value, a potential experiment wide effect existed. A potential timing of insemination effect could have been present which lead to Experiment 3.

Ideally in this study, three separate treatment groups would have been used to determine differences in stress (via capture, rectal palpation) and ultrasound effects by having a strictly control group where the cattle were hyperstimulated, inseminated and not handled until embryo collection. The two treatment groups would have consisted of a frequent rectal palpation and ultrasound group and another group of cattle that were subjected to chute processing which would have delivered a chute stress effect.

Experiment 3

Experiment 3 was conducted to determine if timing of insemination affected the subsequent sex ratio of embryos collected from hyperstimulated beef cattle. The same selection criteria were imposed and cows were used in this final experiment. The same hyperstimulation protocol was followed with the exception that the FSH dosage was again decreased (total dose decreased by 5 mg). The cattle were randomly divided into 0 hour and 12 hour groups. All cattle received GnRH at the onset of estrus to standardize time from estrus to ovulation. Zero Hour

cattle were inseminated at first observed estrus and the 12 Hour group was inseminated 12 hours after first observed estrus. There was a strong trend ($p=0.06$) that the number of transferable embryos collected was lower in the 0 Hour group suggesting a possible decrease in fertility when cattle are inseminated at the onset estrus only in an embryo collection protocol. These data could potentially have an effect on commercial application in an embryo collection program. Many commercial protocols mandate inseminate at estrus and then once or twice subsequently. More research is needed to determine if fertility is reduced in the 0 hour insemination because many collections are performed with sexed semen or expensive semen, so if fertility is affected markedly then a 0 hour insemination may not be cost effective. Because of the decreased fertility in the 0 hour group and the small number of transferable quality embryos collected, no statistical comparison could be drawn between the 0 hour and 12 hour groups when comparing resulting sex of the embryos. However, when 0 hour and 12 hour embryos were combined, the sex ratio did not differ statistically from the expected ratio of 50:50.

Sex ratio in all experiments was independent of developmental stage of the embryo collected. Also, sex was not determined for the degenerative embryos. The sex of the degenerates has no commercial application but could be scientifically relevant if a significant difference in sex ratio is present in the embryos which were no longer viable.

A commonality in all three experiments was the use of a single ejaculate from a single bull. Many sex ratio altering technologies, sexed semen included, have been shown to be bull to bull dependent. A single bull was used in these experiments to lessen the variation from several bulls; however, this potentially subjected all experiments to a bull effect from the use of this single bull. More research is needed to determine if insemination after frequent rectal palpation and ultrasound are impacted by the use of semen from different bulls.

Alternate Studies

Prior to *in vivo* studies, several *in vitro* experiments could have been completed to determine the direct effect of ultrasound on semen parameters. Much research would be needed to determine the frequency and delivery method of the sonication to treat the semen. Some novel research was initiated by determining whether the sound energy would pass through the straw of semen. It was determined that the ultrasound waves did not penetrate the straw because black space could be visualized on the ultrasound monitor indicating that the energy had been disrupted and did not penetrate the straw. Alternative methods to deliver ultrasound energy to the semen would be to cut the straw open and place in a container that allowed ultrasound energy to pass through. Once a proper method of ultrasound energy was determined, research would be needed to determine if different frequencies and different exposure times affected semen parameters differently, or at all. Semen parameter changes could be measured by sonicating the semen and simply visualizing motility changes. Software programs also exist to quantify the actual change in percent and speed in motility. Other studies could be conducted to determine if ultrasound affects different sperm cells differentially by studying the effects of sonication on sexed semen. Since male sperm cells contain less DNA and have a lower mass, it is theorized that ultrasound may potentially have a more adverse affect on the Y-sorted semen than X-sorted semen. Artificial hyperactivation followed by ultrasound could potentially elucidate whether or not hyperactivation and subsequent mobility and speed were affected by the direct effect of the ultrasonic energy.

If an effect was determined to exist, subsequent research could be initiated to determine if ultrasound skewed sex ratio in *in vitro* produced embryos. Fertility and sex of the resulting embryos could be determined by sonication of the semen followed by *in vitro* fertilization and

maturation. Here, a clearer difference might be seen by using sexed semen and seeing if there is a difference in fertility, by measuring the number of embryos formed and cleave to mature. Potentially, the ultrasound affects the fertility of the different sex bearing sperm cells and could sway sex ratio towards female. If a significant effect was determined to exist in *in vitro* studies, then more animal trials would be warranted.

Future Research Direction

There are several potential directions for this research to take in the future. The most interesting question raised by these experiments is the decreased fertility in the cows inseminated at 0 hours following hyperstimulatory treatment. Further research is needed to determine whether or not fertility is actually decreased. An effect that drastic has large commercial implications to the embryo collection and transfer business. A larger study is needed to conclude whether insemination at 0 hour is economically and genetically beneficial to a producer. Along with this research, a larger timing of insemination study is needed to determine whether the interval from standing estrus to ovulation (regulated by administration of GnRH at estrus) effectively skews sex ratio. Larger cow numbers and a more varied window for time of insemination is needed.

Another direction for future research is to further evaluate stress on sex ratio in cows and heifers. There are many different stressors that could potentially sway sex ratio. Using a reliable method to effectively measure stress is important. Cortisol assays from blood serum will determine varying stress levels; however, it is important to note that blood collection itself can cause a spike in blood cortisol levels, so measuring stress by cortisol may have limited usefulness in cattle and can also be confounded by time of day. Cattle could potentially be conditioned to handling to decrease the latent spike in blood cortisol from the simple act of blood collection. There are also other methods available to measure cortisol levels in mammals, such as

fecal and salivary measurements. These applications of cortisol measurement could be limited to chronic stress and make acute stress measurements more difficult.

If the ultrasound and rectal palpation deliver significant stress to alter sex ratio, it is necessary to determine if the stressor is more importantly delivered by the rectal palpation, the ultrasound and manipulation of the reproductive tracts or if both act together. It is important to note that ultrasound of the reproductive tract is not necessarily limited to the reproductive tract in a cow. Other organs, such as the kidneys and by default the adrenal glands, lie very close to the reproductive tract. Future studies could also be conducted to determine if sonication of the kidneys and the adrenals stimulate a stress response and alter sex ratio in resulting embryo production. How stress affects cows versus heifers would also be an important question to answer. If age of donor combined with addition of an acute stressor could trigger changes in sex ratio, there would be potential commercial application.

Future studies could also be conducted to determine if a difference exists between the expected sex ratio from embryos collected from cattle that have undergone hyperstimulatory treatment and single ovulating cows. If stress does cause skewing of the sex ratio, then potentially the act of hyperstimulation is stressful enough to cause a shift in sex ratio of embryos collected from hyperstimulated cows.

CHAPTER SIX

SUMMARY/CONCLUSIONS

The purpose of this study was to determine the effects of ultrasound around time of ovulation on sex ratio of embryos collected from hyperstimulated cows. In the preliminary study (Experiment1), cattle (n=4/group) were synchronized using ultrasound-guided follicular ablation, hyperstimulated and either subjected to ultrasound every 4 hours for 40 hours around time of ovulation or not subjected to ultrasound but processed through the chute. In Experiment 2, heifers (n=7/group) were synchronized using ultrasound-guided follicular ablation, hyperstimulated and either subjected to ultrasound every 4 hours for 40 hours around time of ovulation or neither subjected to ultrasound or chute stress. In these studies, ultrasound did not effectively skew sex ratio when comparing the Treatment group to the Controls. However, when combined, the sex ratio was significantly different when compared to the expected ratio of 50:50. These data suggest a possible timing of insemination effect when the interval from the onset of estrus to ovulation is regulated by administration of GnRH and that this may be important in sex ratio. The possible timing of insemination effect could have potentially outweighed or muted the ultrasound effect.

Experiment 3 was conducted to determine if timing of insemination impacted sex ratio of resulting embryos collected from hyperstimulated cows. Cattle (n=6/group) were synchronized using ultrasound guided follicular ablation, hyperstimulated and either inseminated at 0 hours relative to the onset of estrus or at 12 hours from the onset of estrus. All cattle received GnRH at the onset of estrus. There was a trend that insemination at only 0 hours after the onset of estrus reduced the number of transferable quality embryos collected from hyperstimulated cows.

Statistical inference could not be determined with the sex ratio difference between the groups because there were too few embryos collected from the 0 hour group.

Appendix A

Embryos from Experiment 1

Embryo #	Cow #	T/C	Stage/Grade	Sex
1	N742	T	6-1	M
2	N742	T	5-1	M
3	N742	T	5-1	F
4	N742	T	4-2	M
5	N742	T	16-32 cell	F
6	S715	C	7-1	F
7	S715	C	7-1	F
8	S715	C	6-1	M
9	S715	C	6-2	F
10	S707	C	6-1	F
11	S707	C	6-1	M
12	S707	C	6-1	F
13	S707	C	6-1	M
14	S707	C	5-1	F
15	S707	C	5-1	F
16	S707	C	5-1	M
17	S707	C	5-1	M
18	S707	C	5-1	M
19	S707	C	4-1	F
20	S707	C	4-2	F
21	S707	C	4-3	F
22	S707	C	4-3	M
23	S707	C	16-32 cell	M
24	S707	C	8 cell	F
25	S718	T	7-1	M
26	S718	T	6-1	F
27	S718	T	6-1	M
28	S718	T	6-1	F
29	S718	T	6-1	F
30	S718	T	6-1	M
31	S718	T	16-32 cell	M
32	S718	T	16-32 cell	F
33	S718	T	8 cell	F
34	S718	T	8 cell	F
35	S718	T	8 cell	F
36	S718	T	8 cell	F
37	R314	C	6-1c	F
38	R314	C	6-1	M

39	R314	C	6-1	F
40	R314	C	6-1	F
41	R314	C	5-2	M

Embryos Collected in Experiment 2

Embryo #	Cow #	T/C	Stage/Grade	Sex
1	S105	T	7-1	F
2	S105	T	6-1	F
3	S105	T	6-1	M
4	S105	T	5-1	F
5	S105	T	4-1	F
6	S105	T	4-1	M
7	S105	T	4-1	F
8	S105	T	4-2	F
9	R715	T	7-1C	F
10	R715	T	6-1C	M
11	R715	T	4-2	F
12	R715	T	4-2	F
13	R715	T	8 cell	F
14	R314	T	5-1	M
15	S707	T	7-1	M
16	S707	T	7-1	F
17	S707	T	7-1	F
18	S709	C	4-2	M
19	S709	C	8 cell	F
20	S718	C	6-1	F
21	S718	C	6-1	F
22	S718	C	5-1	M
23	S718	C	5-1	F
24	S718	C	5-2	M
25	S718	C	5-2	F
26	S718	C	4-3 deg	F
27	S718	C	8 cell	F
28	S721	T	7-1	F
29	S721	T	7-1	F
30	S721	T	7-1	F
31	S721	T	7-1	F
32	S721	T	6-1	F
33	S721	T	6-1	M
34	S721	T	6-1	M
35	S721	T	6-1	M

36	S721	T	5-1	F
37	S721	T	5-1	M
38	S721	T	5-1	F
39	S721	T	5-2	F
40	S721	T	4-1	F
41	S721	T	4-1	F
42	S721	T	4-1	F
43	S721	T	4-2	F
44	S721	T	4-2	M
45	S721	T	4-2	M
46	S721	T	16-32 cell	F
47	S721	T	8 cell	F
48	R020	C	5-1	F
49	R107	T	5-1	F
50	R107	T	4-2	M
51	R107	T	4-3	F
52	R107	T	4-3	M
53	R107	T	4-3	M
54	R107	T	16-32 cell	F
55	R107	T	16-32 cell	F
56	R107	T	16-32 cell	F
57	R107	T	16-32 cell	F
58	R107	T	16-32 cell	F
59	R107	T	16-32 cell	F
60	S106	T	5-1	F
61	R041	C	6-1C	F
62	R041	C	6-1C	M
63	R041	C	6-1	M
64	R041	C	6-1	F
65	R041	C	6-1	F
66	R041	C	6-1	M
67	R041	C	5-1	F
68	R041	C	5-1	M
69	R041	C	5-2	F
70	R041	C	5-2	F
71	R041	C	4-1	M
72	R041	C	4-1	M
73	R041	C	4-2	F
74	R041	C	16-32 cell	F
75	R041	C	8 cell	F
76	R041	C	8 cell	F
77	R716	C	7-1C	F
78	R716	C	7-1C	F

79	R716	C	6-1	F
80	R716	C	5-2	F
81	R716	C	4-3	F
82	R103	C	5-2	F
83	R103	C	5-2	F
84	R103	C	5-2	M
85	R103	C	5-2	F
86	R103	C	4-1	F
87	R103	C	4-1	F
88	R103	C	4-1	F
89	R103	C	4-1	M
90	R103	C	4-1	F
91	R103	C	4-2	M
92	R103	C	4-2	F
93	R103	C	4-2	F
94	R103	C	4-2	F
95	R103	C	4-2	F
96	R103	C	4-2	F
97	R103	C	4-3	F
98	R103	C	8 cell	F
99	R103	C	8 cell	F
100	R103	C	8 cell	F

Embryos Collected in Experiment 3

Embryo #	Cow #	T/C	Stage/Grade	Sex
1	N731	12	32 Cell	-
2	N731	12	4-1	M
3	N731	12	4-2	M
4	K715	12	5-2	M
5	K715	12	4 Cell	-
6	K715	12	5-2	M
7	K715	12	8 Cell	-
8	K715	12	4-1	F
9	K715	12	4-1	F
10	K715	12	4-2	F
11	K715	12	4-2	F
12	R317	0	2 Cell	-
13	R317	0	4-2	M
14	H124	12	4-1	M
15	H124	12	4-2	M
16	S242	0	8 Cell	-

17	S242	0	32 Cell	-
18	S242	0	4-2	M
19	L753	12	2 Cell	-
20	L753	12	2 Cell	-
21	L753	12	2 Cell	-
22	L753	12	2 Cell	-
23	L753	12	32 Cell	-
24	L753	12	5-1	F
25	L753	12	5-1	F
26	L753	12	5-1	F
27	L753	12	5-1	M
28	L753	12	5-1	F
29	NT	12	6-1	M
30	NT	12	6-1	F
31	NT	12	6-1	F
32	NT	12	6-1	M
33	NT	12	6-1	M
34	NT	12	5-1	F
35	NT	12	5-1	F
36	NT	12	5-2	F
37	NT	12	5-2	F
38	NT	12	4-1	F
39	NT	12	4-2	F
40	NT	12	4-2	F
41	S242	0	16-32 cell	-
42	S242	0	16-32 Cell	-
43	S242	0	5-2	F
44	S242	0	4-2	F

Appendix B

Commercial Application and Cost Analysis of a 5 Day CoSynch Synchronization Protocol

Abstract:

Artificial insemination (AI) is a tool available to progressive cattle producers to assist them in reaching reproductive, genetic and financial goals. Fixed-time AI programs decrease labor costs for estrous detection and ensure every cow is inseminated. Co-Synch + CIDR is a widely used ovulation synchronization protocol within the beef industry. The objective of this experiment was to determine if the shortened 5 Day Co-Synch + CIDR sufficiently increased pregnancy rates to compensate for the added expense and increased labor of an additional injection and additional processing through the chute. Mature, multiparous Angus and Angus-cross cows ranging in age from 3-15 years averaging 550 kg bodyweight were synchronized using the 5 Day Co-Synch + CIDR program. (Day 0: administer 100 μg GnRH and insert CIDR, Day 5: remove CIDR and administer 25 mg PGF2α, Day 5 + 8 hours: second injection of 25 mg PGF2α, Day 8 (72 hours after CIDR removal): administer 100 μg GnRH and fixed-time artificially inseminate). The pregnancy rate achieved using the 5 Day Co-Synch protocol was 53.7%, which was comparable to pregnancy rate (51.3%) achieved within the same herd using a modified OvSynch protocol (Day 0: 100 μg GnRH, Day: 25 mg PGF2α, Day9: 100 μg GnRH, Day 9 +12 hours: fixed-time AI) in the previous year. Although comparable to the other AI systems in efficiency (pregnancy rates) the success of this approach did not compensate for the additional financial and labor input. However, dependent upon management system, this program can sufficiently decrease estrous detection cost and labor associated with an estrous synchronization program to make this fixed-timed AI protocol economically feasible.

Keywords: cattle, Co-Synch + CIDR, estrous synchronization, ovulation synchronization

Introduction:

Artificial insemination (AI) is an important tool available to cattle producers that will facilitate increased genetic gain, propagation of more elite genetics from outstanding bulls, rapid improvement of economic traits, increased efficiency of sire selection and progeny testing and a decreased number of bulls that need to be maintained on a farm.[1] From a management standpoint, for a cow to maintain a yearly calving interval, she must conceive again within 85 days post-calving.[2] AI implementation can reduce the amount of time necessary to breed cows, shorten the calving season and produce a more uniform calf crop at weaning and provide more predictable calving ease.[1,3] Acceptable pregnancy rates using AI are dependent on several management factors such as proper nutrition before, during and after breeding, proper health status, accurate record keeping, organization and planning of the breeding program, estrous detection (if applicable), semen quality, storage and handling, adequate working facilities and skilled technicians.[2] Several uncontrollable factors such as the weather, latitude and daylight can contribute to AI program efficiency as well. AI is currently used to breed 72.5% of dairy cattle, with timed AI (TAI) programs used in 58.2% of dairy operations, and 7.6% of beef cattle in the United States.[4, 5] There are many factors that contribute to the less frequent use of AI in beef cattle operations. Producers list labor, time and cost as the main reasons not to implement AI followed by too difficult or complicated, other various reasons, lack of facilities and lower confidence in effectiveness.[5] However, under the proper management strategy, AI can be used to add value to a calf crop to sufficiently cover added expense. Estrous synchronization can reduce the amount of time and labor needed for estrous detection and AI. The success of estrous synchronization programs rely heavily on proper estrous detection, as the detection efficiency correlates to pregnancy rates following AI or embryo transfer (ET). Synchronization programs

call for estrous detection anywhere from three to eight days. This is labor intensive and in some cases cost prohibitive, as estrous detection costs range from $15-50 per cow per synchronization cycle.[6] There are many factors that influence detection efficiency which directly affect the cost associated with detection. These factors include the days needed for detection, the amount of time allowed for estrous detection per session and the frequency of detection per day. Others factors such as labor availability, facilities and management systems contribute to the ideal frequency and efficiency of detection. Fixed time AI is utilized in an ovulation synchronization program which further reduces the amount of labor needed for estrous detection because cattle are not examined for estrus and this also ensures that every cow is inseminated. Inseminating every cow adds the possible advantage of getting cows pregnant that might not have been observed in estrus with an estrous detection protocol, but do ovulate. AI along with ovulation synchronization facilitates the use of a timed AI program, which are now achieving pregnancy rates comparable to those achieved by estrous detection programs. The used of a CIDR (controlled internal drug release; a progesterone releasing device) can further increase the efficiency of an estrous synchronization protocol by inducing cyclicity in anestrous cows. An alternative method of delivering exogenous progesterone is the feed additive melangesterol acetate (MGA). This method of progesterone delivery is inexpensive (about $0.02/head/day); however, consistent administration is hard to control because of inconsistencies with feeding habits and irregular feed intake. The addition of a CIDR has been shown to increase pregnancy rates by 10%, (58% vs. 48%) using a conventional CO-Synch protocol.[7] A commonly used fixed timed AI program in beef cattle is CO-Synch + CIDR. Average conception rates using this program in post-partum beef cattle are approximately 55% , with reported rates ranging from 31-80%.[7,9-15] The conventional 7 Day Co-Synch +CIDR AI program is initiated by administration of

100 µg gonadtropin-releasing hormone (GnRH, i.m.) on Day 0 along with the insertion of a CIDR. On Day 7 the CIDR is removed and 25 mg prostaglandin F2α (PGF2α, i.m.) is administered. Sixty to sixty-six hours after CIDR removal all cattle receive a second injection of 100 µg GnRH (i.m.) and are fixed-timed artificially inseminated. A modification to the conventional 7 Day Co-Synch + CIDR is to shorten the exogenous progesterone delivery to 5 days and administer two injections of PGF2α. Reported pregnancy rates using this protocol range from 55-80%.[15-17] The 5 Day Co-Synch +CIDR is initiated following the same protocol as the 7 Day Co-Synch +CIDR with administration of 100 µg GnRH (i.m.) and insertion of a CIDR at Day 0. A novel approach to the conventional system is to shorten the 7 Day CIDR interval to 5 days. On Day 5, the CIDR is removed and an initial injection of 25 mg PGF2α (i.m.) is administered. Eight hours following the initial injection of PGF2α a second dose of 25 mg (i.m.) is administered, which is necessary for the success of the shortened protocol. Ideally, the added advantages in terms of increase in conception rates of using the 5 Day Co-Snych +CIDR program should compensate for the added expense of an extra injection and labor associated with an additional chute processing.

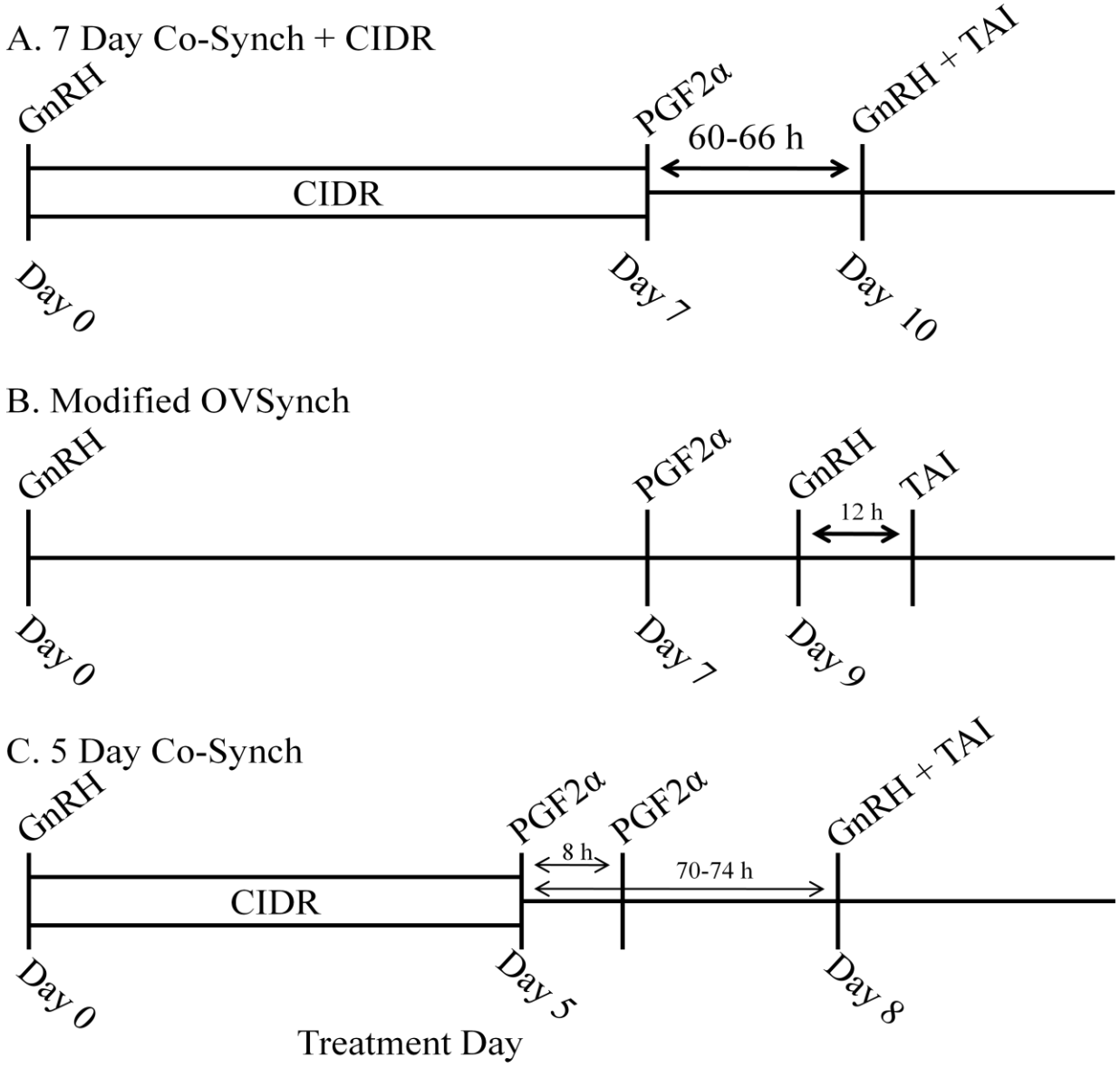
Materials and Methods:

Mature, multiparous Angus, Angus cross and Hereford cattle (n=123) ranging in age from 3-15 years and averaging 500 kg body weight, were administered 100 µg of gonadorelin diacetate tetrahydrate (GnRH; Cystorelin®, Merial, Athens, GA, USA) and a controlled internal drug release (CIDR; Eazi-Breed™ CIDR®, Pfizer Animal Health, New York, NY, USA) was inserted on Day 0. CIDR removal and administration of 25 mg dinoprost (5 ml Lutalyse®, Pfizer Animal Health, New York, NY, USA) occurred simultaneously on Day 5. Approximately 7 hours post CIDR removal, all cattle received a second dose of 25 mg dinoprost. On Day 8, each

cow received 100 µg GnRH and were inseminated with frozen-thawed semen from several bulls; Angus (N=4) or Hereford (N=2). Cattle exhibiting signs of estrus the next day after fixed-timed AI were eliminated. Approximately 2 weeks post timed insemination, bulls were placed with the cows for 45 days. All cows were subjected to transrectal ultrasonic evaluation of their reproductive tracts to determine pregnancy 30 days after insemination. Cattle determined to have conceived from artificial insemination were again evaluated ultrasonically on Day 70 to determine fetal sex.

In another study cattle from a similar origin and environmental conditions were synchronized with a modified Ovsynch™ program. On Day 0 cows (n=152) were administered 200 µg GnRH (im; OvaCyst®; Agri Labs, St. Joseph, MO). On Day 7, cows were given 25 mg PGF2α (im; Prostamate®; Phoenix Scientific Inc., St. Joseph, MO) and on Day 9, given 100 µg GnRH. Any cows that were observed in estrus before Day 9 were eliminated. Twelve hours after the second GnRH, the cattle were randomly divided into Control (N=75) and HeiferPlus™ (HP; n=77) groups.[18] The Control group was inseminated with 0.5 mL of frozen-thawed semen which had been incubated at 37°C for 20 min and the HP group was inseminated with 0.5 mL frozen-thawed semen HeiferPlus™ treated semen. Pregnancy was determined via ultrasonic evaluation 36-38 days post-AI. Fetal sex was determined via ultrasonic evaluation 55-58 days post-AI.

Fig. 1: Timeline for Ovulation Synchronization Protocols



Cost Analysis:

All prices used to calculate $/pregnancy and $/female calf are subject to the assumption that all drugs were purchased from the same vendor (Valley Vet Supply; Marysville, KS[19]) and the prices for injections on a per cow basis were:

GnRH = $2.90/dose

PGF2α= $2.67/dose

CIDR= $10/CIDR

MGA = $0.02/head/day[20]

Chute Processing= $1/head/time through the chute

Chute charges were calculated based on $12.48 per hour wage ($10.00 + 28% benefits) for four individuals for two hours on a hundred cow basis.

Estrous Detection = $15/head/synchronization

Estrous detection fees were based on checking for estrus for 4 days with 2 checks per day for 30 minutes and 2 laborers at $12.48/hour ($10.00 + 28% benefits) on a hundred cow basis.

Results:

The pregnancy rate achieved with the 5 Day Co-Synch + CIDR was 53.7%. The pregnancy rate achieved using the modified Ovsynch™ protocol within the same herd in a previous year was 48% (36/75) for the Control group and 54.5% (42/77) for the HP group. When these two groups were combined the total herd pregnancy rate was 51.3% (78/152). The pregnancy rate for the 5 Day Co-Synch was not statistically different from either the Control group pregnancy rate from the previous study or for the Control and HP groups combined (there was no treatment effect within that study).

Table 1: Comparison of $/AI, $/Pregnancy and $/Female Calf for Different Protocols

Synchronization Protocol	$/AI	$/Pregnancy	$/Female Calf
Traditional 7 Day Co-Synch + CIDR	$21.67	$39.40	*
5 Day Co-Synch + CIDR	$25.34	$47.19	$111.32
Modified Ov-Synch	$12.47	$24.30	$52.65
2 Injection PGF2α	$22.91	$65.43	*
Select-Synch	$23.24	$51.64	*
MGA + 2 Injection PGF2α	$23.55	$33.64	*

Assumptions:
GnRH = $2.90/dose
PGF2α= $2.67/dose
CIDR= $10/CIDR
MGA = $0.02/head/day fed
Chute Processing= $1/head/time through the chute
Estrous Detection = $35/head/synchronization cycle
* Not analyzed in study.

Calculations for $/AI and $/pregnancy for the traditional Co-Synch used a pregnancy rate of 55%. The modified Ov-Synch and 5 Day Co-Synch + CIDR were calculated using data collected, finding 51.3% and 53.7% pregnancy rates, respectively. Calculations were based off field trial studies by Patterson and Smith which found the estrous response rate for the 2 Shot PGF2α, the Select-Synch and the MGA + 2 Shot to be 57%, 67% and 93%, respectively. Also, pregnancy rates were 35%, 45% and 70%, respectively.

Discussion:

Although the conception rates achieved in this study were slightly lower than other published data using the same protocol, [15-17] the pregnancy rate of 53.7% is still an acceptable value for a fixed-timed AI protocol. However, with the increase in cost per AI and cost per pregnancy, this protocol would have ideally shown an increase in pregnancy rates to compensate for the added expense to make this protocol more economically viable. Using a pregnancy rate of 55% for the conventional 7 Day Co-Synch protocol, and all assumptions posted with the chart, within this herd, a pregnancy rate of 64.5% would be needed to make the shortened 5 Day Co-Synch protocol more financially advantageous. On farm, similar pregnancy rates were achieved using the modified Ov-Synch protocol without a CIDR and cost significantly less. The three estrous detection protocols were comparable to the fixed-timed $/AI protocols; however, when comparing the $/pregnancy, an increase in cost was observed when estrous response was lower. The MGA 2 Injection PGF2α was the least expensive in terms of $/pregnancy with excellent estrous response and pregnancy rates; however, supplementing MGA requires a feed or

protein carrier and consistent delivery on a per head basis is difficult to control. Also, estrous detection costs were calculated on the minimal end of the price spectrum (ranges from $15-50 per cow per synchronization cycle) and increased estrous detection intensity would increase costs and labor associated and efficacy of detection would also impact the number of cattle observed in estrus and subsequently artificially inseminated. Labor costs are also impacted by herd size, which should be taken into account when determining which, if any, estrous synchronization program would be the most economically feasible. The larger the herd size, the less viable the more intensive programs that require several trips through the chute will be. Alternatively, a smaller herd size could potentially be cost-prohibitive because price could increase to a $/head amount that is not financially feasible. Herd size should be an important consideration when developing a proper synchronization protocol. In conclusion, the 5 Day Co-Synch protocol produces acceptable pregnancy rates for a fixed-timed AI protocol, but within this herd did not increase the pregnancy rates to sufficiently compensate for the added expense.

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