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Determining the Efficacy of a Commercially Available Post-Thaw Bovine Semen Sexing Kit

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DETERMINING THE EFFICACY OF A COMMERCIALLY AVAILABLE POST-THAW BOVINE SEMEN SEXING KIT

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ABSTRACT

Swaying the bovine sex ratio is beneficial because dairy heifers are significantly more valuable than bull calves, whereas in the beef industry, bull calves are more desirable than females. Currently, there is no inexpensive, reliable, effective method for commercially separating X- and Y- bearing sperm. The objective of these experiments was to determine the efficacy of a commercially available post-thaw bovine semen sexing kit, HeiferPlus^{TM}, which claims to sway the sex ratio in favor of female calves.

Three trials included the insemination of hyperstimulated cows with Control or HeiferPlus^{TM} treated semen, non-surgical embryo collection on Day 7, and a novel combined PCR/ Southern blot assay to determine embryo sex. Overall, 271 embryos were collected and a sex was assigned to 265 of them. Chi square analysis showed that the Control group produced a significantly higher proportion ($p< 0.0005$) of female embryos than the HeiferPlus^{TM} (HP) group (65.0% and 43.0%, respectively). There was no difference in the proportion of transferable versus degenerate embryos and ANOVA showed no difference in the number of ovulations, embryos, and unfertilized oocytes collected from Control versus HP groups. Of the two bulls used in the hyperstimulation studies, one produced an overall higher proportion of females ($p< 0.05$), suggesting a bull effect.

Another trial involved the insemination of cows synchronized via OvSynch® with fetal sexing via ultrasonography between Days 55- 58. Of the 152 cows inseminated, 51.3% (78/ 152) were pregnant at Day 35. Of the cows inseminated with HP treated semen, 54.5% became pregnant and 48.0% of the Controls were pregnant. HP treated cows

resulted in 54.8% male (23/ 42) and 45.2% female (19/ 42) fetuses. Control cows had 52.8% male (19/ 36) and 47.2% female (17/ 36) fetuses at 58 days gestation. Chi square analysis showed no significant difference in the sex ratio proportions. Semen from six bulls was used in this trial, and pregnancy rates among bulls were not different. Computer automated semen analysis (CASA) showed that there was no significant difference in motility or progressive forward motility of Control versus HP treated semen in any of the bulls.

Results of these studies indicated that HeiferPlus^{TM} semen sexing kit did not sway the sex ratio in favor of females in either hyperstimulated or single-ovulating cows. In the hyperstimulated cows, the Control group actually produced a significantly higher proportion of female embryos than the HP group. No differences in embryo production or embryo grade were observed. Further research is needed to investigate the effects of semen incubation or other semen manipulations on the sex ratio of cattle.

DEDICATION

This thesis is dedicated to my Mom and Dad. Their encouragement, support, and continual supply of kitchen-table-talks have allowed me to reach this goal.

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CHAPTER I

LITERATURE REVIEW

Sex Determination

In mammals, sex of the offspring is determined by the presence or absence of the Y-chromosome. An XX genotype results in a female and an XY genotype produces a male. The haploid oocyte from the female always contains an X- chromosome, whereas males produce both X- and Y- bearing haploid sperm, governing which sex will result upon fertilization of the oocyte. It is a common estimation that in most mammalian species, males produce X- and Y- bearing sperm in a 1: 1 ratio, resulting in male or female embryos of the same proportion.

Sperm Capacitation

Capacitation is the process by which sperm cells become competent to bind to an oocyte via molecular changes on the cell surface. First, cholesterol is removed from the sperm cell membrane by albumin proteins present in the female reproductive tract. It is theorized that the removal of cholesterol leads to an increase in pH which eventually allows the sperm to undergo the acrosome reaction (Cross, 1998). Following the removal of cholesterol, other proteins and carbohydrates on the surface are lost, possibly to uncover the sites responsible for binding to the zona pellucida (ZP) of the oocyte. Potassium ions leave the sperm cell, causing a negative membrane potential, and calcium ions enter the cell, activating cAMP production and facilitating fusion events of the acrosome reaction (Visconti, 1995).

The acrosome reaction occurs when the zona binding region of the sperm reacts with and attaches to a protein receptor on the ZP of the oocyte, stimulating the release of enzymes from the sperm that digest the ZP. A second region of the sperm membrane, the acrosome reaction promoting region, also binds to the receptor and causes the sperm plasma membrane to fuse with the acrosomal membrane. This process allows the sperm to penetrate the ZP and enables fusion with the oocyte plasma membrane (Visconti, 1995).

The DNA contained by the sperm is bound by protamine proteins, which are linked by disulfide bonds. These bonds are broken by glutathione, a protein in the oocyte cytoplasm. During syngamy, microtubules connect the sperm and oocyte pronuclei and bring them together. The nuclear envelopes degrade and the chromatin condenses into chromosomes. If an oocyte is fertilized by an X- chromosome bearing sperm, the resulting offspring will be female. If it is fertilized by a Y- chromosome bearing sperm, the offspring will be male.

History and Various Techniques of Altering the Sex Ratio

Most methods of swaying the sex ratio attempt to exploit the differences between X- and Y- bearing sperm. Mass and possibly surface proteins differ between X- and Y- bearing sperm and may provide an approach for separation through techniques such as flow cytometry, sperm surface antigen binding, albumin separation, and pharmacological influence. Timing of insemination relative to ovulation may also affect the sex ratio, as well as semen incubation prior to insemination.

Flow Cytometry

Flow cytometry is a technique by which individual sperm cells can be sorted by mass. Moruzzi (1979) suggested that differences in DNA mass could provide basis for separating X- and Y-bearing sperm. Because of the X- chromosome's larger size relative to the Y- chromosome, X- bearing sperm have approximately 3.7- 4.1% more DNA in their genome, depending upon species (Garner, 1983). This small difference in DNA results in X- bearing sperm having a larger mass than their Y- bearing counterparts.

For flow cytometry to be effective, cellular DNA is stained with a fluorescent dye and a single stream of cells is forced through the flow cell and subjected to a light source. The fluorescence of each cell can be excited into scattering light at a lower frequency than the light source. The scattered light is detected and analyzed, allowing various parameters of the cell to be deduced, such as mass, mass of DNA, proteins, pH, pigments, and enzymatic activity. As sperm cells pass through the flow cell, they can be selectively charged positive or negative based on chromosomal content, and can be deflected into separate paths as the sperm cells are attracted to either the positive or negative plate depending on which charge was applied. The current sperm sorting system permits the separation of six million X- sperm and six million Y- sperm per hour, or if only sorting X- sperm, up to 11 million sperm per hour at a purity of up to 90% depending upon species (Johnson, 2000). After a semen sample is sorted via flow cytometry, the desired sperm cells can be collected and the female artificially inseminated.

Although the accuracy of flow cytometry has been proven in bull semen, the process requires fresh semen and results in a reduction of sperm integrity. Another point of

controversy is the bis-benzimidazole dye, Hoechst 33342, used to stain cells prior to flow cytometry. This stain targets DNA at adenine-thymine-rich regions of the minor groove (Yasui, 2007) and may cause chromosomal abnormalities (Libbus *et al*, 1987). Depending on semen quality, there may be bull to bull variation and sexed semen is only available from selected bulls.

The flow cytometer equipment costs approximately $250,000 and can only sort a limited number of cells per day. The cost of a straw of sexed semen ranges from approximately thirty to fifty dollars for dairy bulls, while the price is quite variable for beef bulls (Hansen, 2006). In addition, there may be variations in the accuracy of flow cytometry between bulls of the same species.

Surface Proteins

Another suggested difference between X- and Y- bearing sperm is surface proteins. It is theorized that selectively binding proteins to X- or Y- bearing sperm will aid in their separation. The H-Y antigen has been studied to determine whether it is preferentially expressed on Y- bearing sperm and whether it could aid in sperm separation: results have been conflicting (Hendriksen *et al*, 1993; Veerhuis *et al*, 1994).

The H-Y antigen was first described by Eichwald and Silmser in 1955 after they observed that male-to-female skin grafts in mice were rejected while grafts within the same sex, as well as female-to-male grafts, usually succeeded. Sills *et al* (1998) treated human sperm with monoclonal immunoglobulin M (IgM) antibodies against the H-Y antigen and incubated the mixture with IgM antibodies appended to paramagnetic beads. The preparation was sorted through exposure to a magnetic field, with anticipated results

that the reactive group would be positive for the H-Y antigen and the non-reactive group would be negative. Flourescent *in situ* hybridization (FISH) showed that 49% of Y-bearing sperm did not express the H-Y antigen. Currently, no reliable sex-specific antigens have been identified on mammalian spermatozoa (Howes *et al*, 1997; Hendrickson, 1999).

Studies using mice have shown that developing X- and Y- bearing sperm express different genes; however, these products may be shared among gametes due to the intercellular bridges formed during spermatogenesis (Hendrickson, 1999). Braun *et al* (1989) used transgenic hemizygous mice with mouse Protamine 1 (mP1) transcriptional regulatory sequences fused to the human growth hormone (hGH) gene to demonstrate that RNA and protein can pass through the intercellular bridges among spermatids. The hGH gene was transmitted to 50% of the developing sperm population, but subsequent immunocytochemical analysis showed that 90% of sperm contained hGH. The level of sharing may actually be greater: of the homozygous controls in the same study, only ninety-two percent were positive for hGH. Caldwell and Handel (1990) also demonstrated that post-meiotic spermatids share mP1 gene products through intercellular bridges.

Albumin Separation

Albumin separation is a technique in which semen is diluted with Tyrode's solution in a 1:1 ratio prior to centrifugation. The sperm pellet is removed, resuspended in Tyrode's solution, and is layered over columns of bovine serum albumin (BSA). This separation technique is based upon progressive forward motility and results in the isolation of Y-

bearing sperm (Ericsson, 1973). Ericsson reported in *Nature* (1973) the isolation of 85% human Y- bearing sperm with up to 98% progressive forward motility. In 1975, Ross *et al* attempted to repeat the same experiment, but results failed to support Ericsson's conclusions. Another study attempted albumin separation using bull semen and samples were analyzed using flow cytometry. Results showed that the ratios of X- and Y- bearing sperm were the same for the treatment group and the controls (Beal, 1984).

A study conducted in Berkeley, CA showed that 71% male offspring were produced out of 1,407 human births in sixty-five fertility clinics in the US and abroad through an albumin separation technique followed by intrauterine insemination on the presumed day of ovulation (Beernink, Dmowski, and Ericsson, 1993). In contrast, an experiment with rabbits found that the sex ratio was unaltered by the separation of sperm through albumin gradient (Zavos, 1985). It was previously believed that this separation process resulted in a sperm sample with a higher representation of Y- bearing sperm (Hafez, 1987). A group in Hong Kong determined through fluorescent *in situ* hybridization (FISH) that human sperm separation did not increase the concentration of Y- bearing sperm, rather, they postulated that serum albumin inactivated X- bearing sperm (Rose, 1998). Because the mode of action of albumin separation is unknown and results are contradictory, there is justified doubt over its effectiveness and use.

Drugs and Environmental Factors

Exposure to certain drugs and toxins has been shown to alter the sex ratio. Davis *et al* (1998) found a decrease in male births in industrialized countries. They concluded that

the reduction in male births should be viewed as a sentinel health event that could be linked to environmental factors such as pollution.

Dibromochloropropane (DBCP), a toxic nematocide used extensively until the 1960's, has been shown to induce testicular dysfunction in workers subjected to high levels of exposure. Pregnancies conceived during exposure to DBCP resulted in 35% males whereas those same couples produced 53% males during the pre-exposure period (Potashnik and Porath, 1995).

Clomiphene citrate (trade names: Clomid®, Serophene®, Milophene®) is a non-steroidal estrogen antagonist used to treat infertility by inhibiting estrogen binding by receptors on the anterior pituitary. This leads to increased production of FSH followed by LH and results in higher rates of ovulation. The use of clomiphene citrate also resulted in significantly fewer males at birth. Jarrell (2002) studied the drug's effects on the sex ratio and, although the exact mechanism is unknown, Jarrell hypothesized that the altered sex ratio was due to its synergistic actions on the estrogen receptors of the female. Sampson (1983) found that out of eighty-nine clomiphene citrate induced ovulations followed by intrauterine insemination, 53.9% resulted in females. Silverman *et al* (2002) used clomiphene citrate in conjunction with albumin separation prior to intrauterine insemination and reported 51.9% females compared to the control group which had 48.6% females.

Dioxin is a chemical produced as a byproduct of the manufacturing of Agent Orange (2, 4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid), which is an herbicide used extensively during the Vietnam War to defoliate plants and trees. An

article published in *Epidemiology* (Michalek *et al*, 1998) reported that dioxin exposure lead to significantly more female offspring. Data from the Air Force Health Study examined offspring of veterans of Operation Ranch Hand, which is the unit that handled Agent Orange during the Vietnam War. Men who conceived children (n= 181) less than one month postservice resulted in 56.9% females. After an industrial accident in Seveso, Italy, nine families in which both parents were exposed to dioxin resulted in one-hundred percent female children (n= 12). Data on the health of the resulting children was not reported. The National Toxicology Program classified dioxin as a human carcinogen in its Second Annual Report on Carcinogens (1981).

Occupation

Retrospective studies have examined the relationship between certain occupations and the sex ratio of offspring. In 1982, *The Lancet* published a study by W.R. Lyster examining the offspring sex ratio of Australian abalone divers. Of the fifty-eight participants in the study, there were eighty-five daughters (65.4%) and forty-five sons (34.6%) between them, all conceived after their fathers had become divers. Other studies have investigated the offspring sex ratio of pilots and astronauts exposed to high G forces. Pilots exposed to higher G forces had sixty percent female children, while those considered to work at low G forces showed no statistical difference in their offspring's sex ratio (Little, 1987). After administering a comprehensive questionnaire to one thousand German pilots, Goerres and Gerbert (1975) of the German Air Force Institute of Aviation Medicine concluded that the sex ratio of helicopter pilots significantly shifted in favor of female offspring after the one thousandth flying hour.

Timing of Insemination

Another factor that may influence embryo sex is the timing of insemination relative to ovulation. Mammalian sperm can reach the oocyte quickly relative to insemination but may not be competent to fertilize the egg. They acquire competence as they move towards the ampulla, but will become unable to fertilize if they remain in the oviduct for an extended period of time (Eisenbach, 1995). It has been observed that uncapacitated sperm temporarily bind to the membranes of the oviducts in the isthmus and are released upon capacitation (Smith, 1998). This event slows down capacitation and lengthens the life span of the sperm, maximizing the probability of sperm being present in the ampulla to fertilize the egg even if insemination does not occur at a time favorable to ovulation (Gilbert, 2003).

OvSynch® (Pursley, 1995) is an ovulation synchronization protocol in which Gonadotropin Releasing Hormone (GnRH) is administered on Day 1, prostaglandin is given seven days later, followed by a second injection of GnRH on Day 9. Pursley *et al* (1998) conducted a study that synchronized cows via OvSynch® and examined the timing of insemination relative to ovulation. Cows were inseminated at 0, 8, 16, 24, or 32 hrs relative to the second injection of GnRH. Cows bred at 0 and 32 hrs had the highest percentage of female offspring (61.0% and 65.0%, respectively), with the 0 hr group having the lowest pregnancy loss (between Day 35 and parturition) and the 32 hr group having the highest.

A 2003 study by Martinez *et al* examined the timing of insemination relative to the onset of estrus. They found that significantly more female calves (73.1%) resulted from

inseminations performed from eight to eighteen hours following first observed estrus. A study in sheep found that more females resulted from inseminations five hours prior to ovulation and more males resulted from inseminations occurring five hourrs after ovulation. The *New England Journal of Medicine* published a study (Guerrero, 1974) in which 1,318 conception cycles were evaluated to determine if basal body temperature at insemination impacted the sex ratio of human births. In natural conception, male births were most common (68.0%) when insemination occurred six days prior to the temperature shift, decreasing to 44.0% on the day of the shift. In artificial insemination, the trend was the opposite (Gutierrez-Adan, 1999).

Other studies have found no effects of timing of insemination on sex ratio. Rorie *et al* (1999) found no difference in sex ratio of calves resulting from inseminations either 20 hr or 10 hr prior to the expected time of ovulation. In sows that had recently farrowed and weaned, Soede (2000) demonstrated that the sex ratio was unaffected when sows were inseminated at four hour intervals from the onset of estrus until ovulation. A study published by the *New England Journal of Medicine* (Wilcox, 1995) showed that the timing of sexual intercourse in relation to ovulation had no influence on the sex of the child, whereas a study at Johns Hopkins reported that conception occurring close to ovulation resulted in a significantly lower proportion of male births (Gray, 1991).

Semen Incubation

A study of *in vitro* produced bovine embryos showed that a 24 hr semen incubation period resulted in significantly more female hatched blastocysts when compared with 0 or 6 hr semen incubation periods (Lechniak, 2003). Watkins *et al* (1996) found that human

X- bearing sperm had significantly higher percentages of motility, rapid progression, and hyperactiviation after 24 hr incubation when compared to Y- bearing sperm. They also had faster curvilinear and straight line velocities. Because no differences in sperm head or tail shape have been found, the mechanism is unknown.

Other Physiological Factors

Oviductal Proteins

Other studies have shown that an equal ratio of X- to Y- bearing sperm in a semen sample can result in a greater percentage of offspring of one sex. Recently, oviductal proteins have been shown to interact and possibly influence gamete and embryo development. Oviduct-specific glycoproteins (OSG) and osteopontin have been shown to affect sperm capacitation, gamete binding, fertilization, and embryo development (Killian, 2004). It is possible that other secretions of the oviduct could influence the sex ratio through preferential binding of one sperm type to the oocyte or through a mechanism operating on the embryo prior to implantation. Of the total amount of ovine embryos produced *in vivo*, a higher percentage of male embryos are represented, yet the sex ratio at birth is still 50:50 suggesting that more male embryos are lost prior to or just after implantation (Catt, 1997). A study with *in vitro* produced bovine embryos found that culture systems using synthetic oviduct fluid medium with fetal calf serum resulted in significantly higher rates of male blastocyst survival when compared to other culture systems, although the mechanism is not understood (Gutiérrez-Adán, 2000).

Cervical Mucous

Martin (1997) found that the sex ratio was altered depending on the length of the follicular phase in women, with longer follicular phases resulting in more females. He suggested that properties of cervical mucous may differ depending on the length of the follicular phase, and that these differences may select one sperm type over another. In cows, Wehner *et al* (1997) utilized the OVATEC intravaginal probe to determine the conductivity of cervical mucous as a gauge for insemination times in order to sway the sex ratio. They found that significantly more heifer calves resulted from inseminations that occurred when impedance values were low (35- 45; ~20 hrs prior to ovulation) and significantly more bull calves resulted when impedance values were high (50- 70; ~10 hrs prior to ovulation).

Sex Determination in Non-Mammalian Species

Current methods of manipulating the sex ratio in mammals may not be applicable to other organisms. In avian species, the male is homogametic, designated ZZ, while the female is heterogametic, or ZW. In *Drosophilia*, the Y- chromosome does not generate a male while the lack of two XXs does. In other insects, such as bees, fertilized eggs will become females while unfertilized eggs develop into males (Manolakou, 2006).

In most reptiles, sex is dependent upon the incubation temperature of the eggs, not by sex chromosomes deposited into the egg by a male. In species that display temperature-dependent sex determination, males and females have identical karyotypes (Modi and Crews, 2005). Turtle eggs incubated at lower temperatures (26°C) yield males, while those incubated at higher temperatures (33°C) yield females. Alligators are the opposite,

with lower temperatures producing females and increased temperatures resulting in males. Differentiation of gonads into ovaries or testes depends upon the incubation temperature of the eggs during a period of development in which the embryo is thermosensitive. It is believed that temperature affects the undifferentiated gonads, since they are the site of estrogen synthesis and aromatase activity. Aromatase is the enzyme responsible for converting testosterone into estrogen and its activation or inhibition plays a significant role in sexual differentiation, with increased activity leading to females and decreased activity resulting in males (Pieau, 2004).

SRY Gene

The sex-determining region of the Y- chromosome, or SRY gene, was discovered in 1990 and is located near the tip of the short arm. It is an intronless region that encodes for a nuclear factor-like protein with a central high mobility group (HMG) box (Cheng, 2001; Nagai, 2001). It is called the testis determining factor (TDF) and is responsible for differentiation of the testis, which secrete anti-Mullerian hormone (AMH) from Sertoli cells and testosterone from Leydig cells. Together, these hormones stimulate masculinization of the fetus. If the SRY gene is absent, the gonad differentiates into an ovary. Other genes that play a role in sexual differentiation are Sox9, an autosomal gene that aids in testis formation and is possibly turned on by SRY, and Steroidogenic Factor 1 (Sf1), a transcription factor which may also be regulated by SRY to increase levels of AMH (Gilbert, 2003). In the bovine, expression of the SRY gene can be detected as early as the four to eight cell embryo through rt- PCR (reverse transcriptase-polymerase chain

reaction) (Gutiérrez-Adán, 1997). Currently, no target gene of SRY protein has been identified (Polanco, 2007).

Selecting for Sex at the Embryo Level

Following semen sexing via flow cytometry, a single sperm cell of the desired sex can be injected directly into the oocyte *in vitro* in a process called intracytoplasmic sperm injection (ICSI). This technique assures an embryo of a chosen sex, but is invasive, inefficient, and can be damaging to the oocyte. *In vitro* fertilization (IVF) is a process in which the gametes are collected and fertilization takes place in culture. The resulting embryos can then be transferred into a recipient or frozen for later use. Studies describe a higher number of male offspring resulting from IVF, possibly due to the rapid early division and growth of male embryos relative to female embryos. Embryos at a more advanced stage in development are more likely to be selected for transfer (Gutiérrez-Adán, 2000). Kochhar *et al* (2001) theorized that because the Y- chromosome may contain more genes that function as transcription factors, development could be accelerated, whereas the X- chromosome contains genes that code for rate limiting steps required in embryonic developmental pathways.

PCR

The sex of embryos can be determined prior to embryo transfer by polymerase chain reaction (PCR), a method first described by Kari Mullis (1986). This technique is useful because it requires only one or two blastomeres, leaving a viable embryo able to be transferred into a recipient or frozen for later use. In PCR, short, specific sequences of DNA can be amplified exponentially in three steps: denaturing, annealing, and extension.

First, DNA is denatured through exposure to high temperatures in order to break the hydrogen bonds and separate the double strands. The temperature is then lowered to allow primers, which are short, single strands of DNA complementary to the beginning and end of the sequence to be amplified, to anneal to the DNA template. A Taq DNA polymerase then fills in the missing complementary strands with supplied nucleotides. The desired sequence doubles with each PCR cycle, allowing for easier identification and analysis.

To determine the sex of a mammalian embryo, a DNA sequence specific to the Y-chromosome can be amplified and the product can be identified using agarose slab gel electrophoresis. If a visible band is produced by the primer pair, the specific region of DNA is present and the embryo can be identified as a male. Lack of that band would signify lack of a Y- chromosome, indicating a female embryo. PCR was first used for genetic diagnosis in bovine blastocysts in 1988 (King and Wall) and is now a widely used method of sexing bovine embryos (Weikard, 2001; Bredbacka, 1995; Alves, 2003; Park, 2001; Cheng, 2001). Although it is sensitive and specific, it often imparts inconsistent results when assessing samples with limited template, especially when copies of target genes are unequal. Almodin *et al* (2004) reported successful sex determination in 82.0% of embryos, using bovine embryos as models for human preimplantation genetic diagnosis (PGD). A retrospective study conducted by Shea (1999) examined the use of PCR for sexing 4,183 embryos in a commercial bovine embryo transfer program. Results showed that sex could successfully be determined in 90.0% of samples; failures at sexing were attributed to malfunctions of specific PCR reagents, contamination, and insufficient

DNA template. Because PCR assays for embryo sexing have been optimized in order to amplify samples with small amounts of DNA, they are also sensitive enough to amplify contamination by a single DNA molecule.

Southern Blot

Southern blot is a sensitive method of determining the presence of specific DNA sequences. DNA is treated in an alkaline solution, causing it to separate into single strands and then immobilized onto a membrane. The DNA can be crosslinked to the membrane by allowing it to dry or exposing it to ultraviolet radiation. A probe is created, which is an isolated DNA molecule complementary to the entire sequence of interest, labeled with a fluorescent tag or chromogenic dye. The membrane is exposed to the probe and allowed to hybridize. The hybridization pattern can be visualized on x-ray film by radiography or by color development, showing which DNA samples contain the sequence of interest.

A 2006 study by Shekhar *et al* compared a Southern dot blot technique to PCR for detection of white spot syndrome virus in shrimp tissues. Each technique was used independently, and researchers found that PCR analysis was more sensitive than Southern blotting of crude DNA extracts, although the blots were able to detect the presence of genes in some tissues (eye and eye stalk) that PCR could not. In a clinical study, Schiffman *et al* (1991) also compared Southern blot to PCR in order to identify human papillomavirus DNA and found that PCR detected more positive samples than Southern blot.

Another study combined PCR with Southern blot by amplifying bovine Herpesvirus 4 (BHV-4) from experimentally infected calves (Boerner *et al*, 1999). Using PCR alone, they could detect a lower limit of 10 fg of BHV-4, whereas by blotting PCR product, they were able to detect as little as 1 fg: a ten-fold increase in sensitivity.

Trypsin/ Percoll Semen Washing Method

A novel method of semen washing used a trypsin-percoll density gradient to wash sperm cells (Loskutoff, 2005). Trypsin is an enzyme naturally produced by the pancreas that the International Embryo Transfer Society recommends to remove viruses and pathogens from livestock embryos without affecting their viability (Stringfellow, 1999). Loskutoff's method combines a trypsin wash with a percoll density gradient and was devised in an attempt to rid human sperm cells of HIV viruses that may contaminate semen (Appendix C). Silva, Solana, and Castro (1998) reported that trypsin treatments on frozen-thawed bull semen, although effective against bovine rhinotracheitis virus, damaged sperm cell plasma membranes. Furimsky *et al* (2004) determined that percoll gradient centrifugation resulted in mouse sperm with significantly higher fertilization ability, as determined by sperm-zona pellucida binding. Loskutoff reported (2005) that the trypsin/ percoll density gradient had no detrimental effects on fresh or frozen-thawed human semen.

CHAPTER II

INTRODUCTION

Benefits of Manipulating the Sex Ratio

Swaying the sex ratio is beneficial in both agriculture and human fertility treatments. In the dairy industry, female calves are significantly more valuable than male calves because of their ability to produce milk, whereas in the beef industry, male calves are more desirable due to their rapid growth and efficient meat production. In swine production, males are undesirable due to boar taint, which is an undesirable flavor and odor associated with intact males and also, females reach market rate quicker than males. Niche markets, such as endangered species, pets, or laboratory animals may also prove to be useful applications for sex selection (Johnson, 2000). To date, there is no effective, inexpensive method for manipulating the sex of offspring.

Ethical Concerns

While the purpose of altering the sex ratio in livestock is to reach financial goals, in human assisted reproduction, couples may want a child of a certain sex in order to avoid sex-linked genetic disorders, which are inherited through one of the sex chromosomes. Because males have only one copy of the X- chromosome, any gene present on the X-chromosome will be expressed, even if recessive. Hemophilia, Duchenne muscular dystrophy, and Fragile X syndrome are examples of recessive sex-linked diseases found in males. Reasons to want a child of a certain sex may be for family balancing or to avoid sex-linked diseases; however, in India and China, there is a strong politically-driven

preference towards males. A recent census in India showed that the male: female ratio of children under six years of age was 1000:927. In Haryana state, the ratio was smaller, with 1000:820 (Registrar General, 2001). In areas of China where the "one child" policy is mandated, the sex ratio is 117:100, but in rural areas of the country where multiple children are permitted, the sex ratio is balanced (Plafker, 2002; Institute of Philosophy, 2004). In most other countries, including the US, Canada, and the UK, census data shows that no sex preference exists (Royal Commission on New Reproductive Technology, 1993).

Rationale

Currently, there is no effective, reliable, inexpensive method for commercially separating X- and Y- bearing sperm. Although flow cytometry is the most effective technique to date, it is expensive and may cause damage to sperm cells. No sperm cell surface proteins have been isolated that are specific to either X- or Y- bearing sperm and results of albumin separation studies have been conflicting. Although certain toxins and environmental factors appear to have an effect on the sex ratio, usually favoring females, the mechanisms are not well understood and the risks outweigh the benefits. Intracytoplasmic sperm injection, *in vitro* fertilization, and embryo sexing require expensive equipment and supplies, thereby limiting them mainly to human fertility treatments.

HeiferPlus™

In 2005, Emlab Genetics (Arcola, IL) began producing a post-thaw bovine semen treatment product called HeiferPlus™ (HP) that attempted to sway the bovine sex ratio in

favor of females. The manufacturer published internet resources supporting its claim in both hyperstimulated and naturally ovulating cattle (Williams, 2007). Day 7 embryos were collected from fifteen hyperstimulated Angus cross heifers that were inseminated 12 hrs post standing estrus. Using PCR analysis, a male-specific sequence present on the Y-chromosome was probed. Of the fifty embryos collected, 74% were determined to be female (37/ 50): a statistically significant shift in the expected sex ratio of 52/48 (M/ F). In 2006, Emlab Genetics conducted four breeding trials on a total of 58 Holstein heifers. Pooling their data, they found 81.0% heifers (n= 47): significant at the 0.01 level.

Due to the proprietary nature of the product, the ingredients and its mode of action are undisclosed. Although the manufacturer yielded promising results, the studies included no control animals, no reported positive or negative PCR controls, and no independent trials have been reported.

CHAPTER III

EXPERIMENT ONE: HEIFERPLUS™ EFFICACY IN HYPERSTIMULATED COWS INSEMINATED AT FIRST OBSERVED STANDING ESTRUS

Objective

The objective of this study was to evaluate the efficacy of HeiferPlus™ semen sexing kit in hyperstimulated cows artificially inseminated at first observed standing estrus.

Materials and Methods

Estrous synchronization and follicular ablation

On Day 0, cows (n= 10) and heifers (n= 11) were rectally palpated and significant ovarian structures were recorded. They were administered 25mg prostaglandin (IM; ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO) and received a CIDR device (Eazi-breed CIDR; DEC International, NZ). On Day 7, all animals were given 25mg prostaglandin and CIDRs were removed from the cows. CIDRs were removed from the heifers the following day and estrous checks were conducted on all animals every 12 hrs with the utilization of KAMAR® heat detection patches (Kamar, Inc. Steamboat Springs, CO). On Day 6 following observed estrus, follicles greater than 5mm were destroyed via ultrasound-guided transvaginal ablation, 25mg prostaglandin was administered, and CIDRs were inserted.

Superovulation and Artificial Insemination

Forty-eight hours following follicular ablation, FSH treatment was initiated. Cows were treated with a total of 30mg FSH (IM; Folltropin®-V; Bioniche, Belleville, Ontario, Canada) in decreasing divided doses twice daily for 4 days. Heifers were treated with a

total of 20mg FSH in decreasing divided doses twice daily for 4 days. In cows and heifers, CIDRs were removed 48 hrs into the FSH treatment and 25mg prostaglandin was administered. Cows and heifers were randomly assigned to Control or HeiferPlus^{TM} (HP) groups and were inseminated at first observed standing estrus. Control animals were inseminated with 1ml frozen-thawed semen incubated at 37°C for 20 minutes. HP animals were inseminated using 1ml of frozen-thawed, HeiferPlus^{TM} (Emlab Genetics, Arcola, IL) treated semen, prepared as per manufacturer instructions (Appendix B). All semen came from a single ejaculate of the same bull.

Embryo Collection and Freezing

Embryos were collected via non-surgical uterine lavage seven days post AI using one liter of 1x PBS solution containing 15mls BCS (HyClone, Logan, Utah), 50,000u Penicillin, and 50mg Streptomycin (Mediatech, Inc., Herndon, VA). The embryos were graded, transferred to a 4-well plate containing holding media (ViGro Holding Plus; Bioniche), and rinsed. They were then moved to 0.25mls 10% glycerol (ViGro Freeze Plus; Bioniche) for twenty minutes. Embryos from one cow were put into the same straw before slow-rate freezing at a ¯0.5°C/ minute cooling rate. At ¯35°C, the straws were plunged into liquid nitrogen at ¯196°C, where they were stored until PCR analysis.

PCR analysis

Embryo straws were thawed in a 30°C water bath for thirty seconds. Embryos were then transferred to holding media (ViGro Plus) where they were rinsed twice. Each embryo was transferred to a PCR tube containing 3µl Proteinase K (1µg/µl) solution (Fisher Scientific, Fairlawn, NJ). The tubes were incubated at 55°C for 10 minutes to

allow digestion of the zona pellucida and then placed in a 100°C water bath for 10 minutes to inactivate the Proteinase K. The tubes were cooled to room temperature and the PCR reaction mixture was added. A multiplex PCR was performed using two sets of primers (Invitrogen, Carlsbad, CA). A bovine specific primer pair was used to amplify a 219 bp fragment from a bovine 1.715 satellite (forward: 5'- TGA GGC ATG GAA CTC CGC TT- 3'; reverse: 5'- GGT GGT TCC ACA TTC CGT AGG AC- 3'). A 131 bp Y-chromosome fragment was amplified (forward: 5' GAT TGT TGA TCC CAC AGA AGG CAA TC – 3'; reverse: 5' GAA CTT TCA AGC AGC TGA GGC ATT TA -3') (Xu, 2006). PCR mixture: 2.5µl Taq buffer (Promega, Madison, WI), 0.25u Taq DNA Polymerase (Eppendorf, Westbury, NY), 3µl $MgCl_2$ (Promega), 2µl dNTPs (Invitrogen), 9.75µl sterile H_2O, 0.25µl of each satellite primer and 2µl of each Y-primer (Invitrogen). DNA isolated from bovine testis and ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls and a negative control contained no DNA.

PCR was initiated with a cycle of 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 64°C for 30 sec, extension at 72°C for 30 sec, and a final hold at 72°C for 10 min. Three microliters of 6x loading dye (Promega) was added to 12µl of PCR product and analyzed on 1.6% agarose gel containing 3µl EtBr 1% (Fisher Scientific). One band at 219 bp denoted a female embryo while two bands, one at 219 bp and one at 131 bp, indicated a male embryo.

Chi square analysis was used to evaluate differences in proportions, whereas one-way analysis of variance (ANOVA) was used to determine differences in means of ovulations, embryos collected, and unfertilized oocytes collected.

Results

A total of 46 embryos were collected from 17 cows. Four cows were omitted from the study due to failed hyperstimulation (< 2 CLs at time of embryo collection). Embryo sex was determined for 44 of the total samples (95.65%), as two HP samples appeared blank on the agarose gel. Positive and negative controls were consistent between gels.

Figure 3.1. Agarose Gel Following Multiplex PCR Amplification

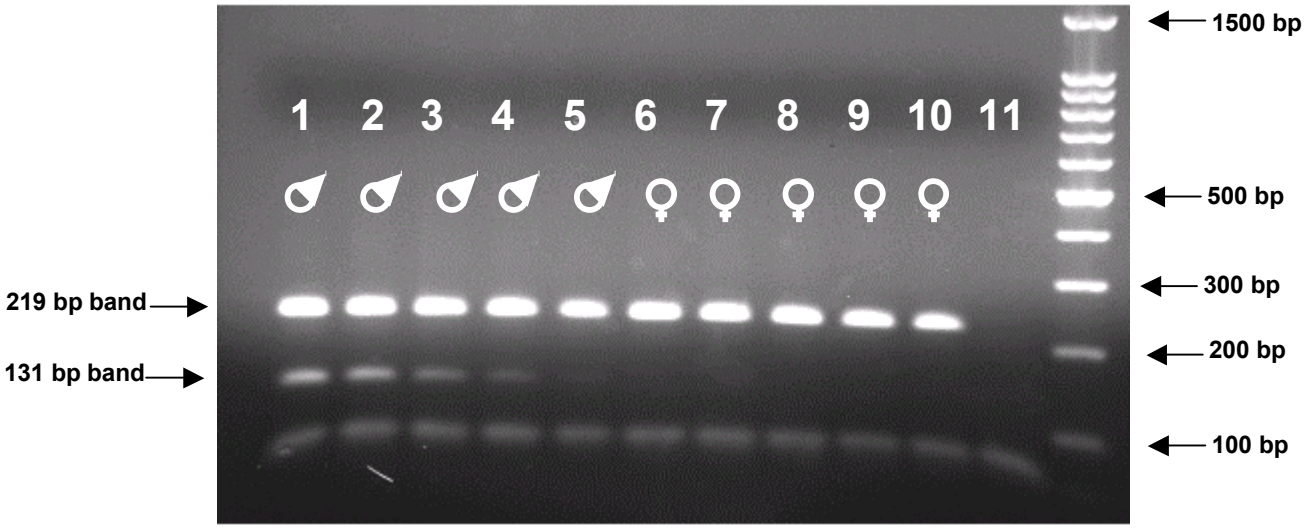


Figure 3.1 shows a picture of a 1.6% agarose gel stained with ethidium bromide. Lanes 1 through 5 show PCR product from male DNA in decreasing concentrations of initial template (2.0ng to 0.1ng). Lanes 6 through 10 show PCR product from female DNA in decreasing concentrations of initial template (2.0ng to 0.1ng). The lanes with one band at 219 bp indicate a female, while lanes with two bands, one at 219 bp and one at 131 bp, denote a male. Lane 11 is the negative control and the 100 kb ladder is in Lane 12.

Table 3.1 shows the total number of ovulations, embryos collected, and unfertilized oocytes (UFOs) for each group, as well as means and standard error for the means. Single

factor ANOVA showed no significant difference in the number of ovulations, embryos collected, or UFOs between Control and HP groups.

Table 3.1. Comparison of Ovulations, Embryos, and UFOs[1] between Groups

	Control (n= 7)	HP (n= 10)	p-value
Total Ovulations[2] (Mean ± SEM)	129 (18.43 ± 1.45)	203 (20.30 ± 0.89)	0.737
Total Embryos (Mean ± SEM)	17 (2.43 ± 0.31)	29 (5.27 ± 0.82)	0.757
Total UFOs[1] (Mean ± SEM)	14 (2.00 ± 0.25)	18 (4.50 ± 0.57)	0.285

[1] UFOs: unfertilized oocytes collected.

[2] The number of ovulations was determined by the number of corpora lutea present at time of embryo collection.

Chi square analysis was used to determine if the proportion of ovulations versus embryos collected was affected by HP treatment and showed no significant difference at the 0.05 level. There was also no difference in sex ratio between the groups (Table 3.2). The HP group produced a significantly higher proportion of degenerate embryos ($p < 0.01$) which were the same proportion of males to females. There was no difference in sex ratio or embryo quality between cows producing greater than five embryos and those producing less than or equal to five embryos.

Table 3.2. Sex Ratio of Embryos by Group as Determined by PCR

	Control (n= 17)	HP (n= 27)	p-value
Number Female (%)	11 (64.71)	13 (48.15)	0.283
Number Male (%)	6 (35.29)	14 (51.85)	

Conclusions and Discussion

Following data analysis, a NCBI BLAST was performed on the male-specific sequence and it was determined that the region of amplification was not male specific. A later attempt was made to amplify a different segment of the Y- chromosome from the previous PCR product.

PCR analysis

PCR product was thawed to room temperature and 5µl was used as template for a multiplex PCR using two sets of primers (Invitrogen, Carlsbad, CA). A segment of bovine β-actin (385 bp) was used as an internal positive control (forward: 5'- CCG AGG ACT TGA TTG TAC ATG G- 3'; reverse: 5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3'). A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCT TCC TTG TGC ACA GAC AG– 3'; reverse: 5' TTA TTG TGG CCC AGG CTT GT-3') (Daneau *et al*, 1995). Primers were diluted to 250pmoles/ µl in TE stock, then diluted 1:10 in DepC H_2O working stock. PCR mixture: 10µl Go-taq (Promega, Madison, WI), 1µl of each β-actin primer and 2µl of each SRY primer. DNA isolated from bovine testis and ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls and a negative control contained no DNA. PCR was initiated with an initial cycle of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec, hold at 72°C for 3 min, and a final hold at 4ºC.

Southern Blot

Southern blots were performed on each of the PCR products. Thirty microliters of double distilled H_2O was added to the PCR products, followed by 6µl 1N NaOH. The reaction was incubated at 37ºC for five minutes. Following denaturation, 20µl of 20x SSPE was added, followed by 200µl 6x SSPE. Using a vacuum-mediated dot blot manifold, samples were added to 6x SSPE pre-soaked nylon membrane (Biobond™-Plus Nylon Membrane; Sigma, St. Louis, MO) over two pieces of Whatman paper. Samples were run in duplicate, along with positive controls ranging from 2.0ng to 0.04ng DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, USA). The membrane was crosslinked twice (Spectrolinker; Spectronics Corporation, Westbury, NY) at the 'optimal crosslink' setting. Membrane hybridization was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK) with overnight hybridization at 55ºC. CDP-Star™ chemiluminescent detection system was used with a 25min exposure on Kodak BioMax Light Film (Kodak, Cedex, France). Membranes were first probed for the SRY gene segment and then stripped by washing in boiling 6x SSPE and 0.5% SDS for 15min, twice. Membranes were then probed for the β-actin segment.

Results of Re-Amplification/ Southern Blotting

Blots from this experiment showed evidence of contamination. While some samples appeared clean, others were questionable. It was determined that DNA from previous samples contaminated the dot blot manifold. Rather than hand-pick the samples the

author felt comfortable assigning a sex, it was decided to omit the entire group from analysis to avoid biases in interpretation.

It should be noted that although the NCBI BLAST reported that the amplified 'male-specific' region was found in female cow liver, both the male and female positive controls in this study showed appropriate bands in the agarose gel. Also following this experiment, the HeiferPlus^{TM} manufacturer recommendations changed from inseminating at time of standing estrus to inseminated 12 hrs post standing estrus.

CHAPTER IV

EXPERIMENT TWO: EFFECTS OF TRYPSIN WASH ON HEIFERPLUS™ PERFORMANCE

Objective

The previous study indicated that HeiferPlus™ did not alter the sex ratio in favor of females in hyperstimulated cows (Chapter III). A novel sperm cleaning method utilizing a trypsin/ percoll gradient (Luskotoff, 2005) had been found to remove viruses and debris from sperm cell surfaces. If the semen sample is cleaned, it is possible that the mode of action of HeiferPlus™ would be improved. The objective of this experiment was to determine if the trypsin/ percoll semen washing method enhances the efficacy of HeiferPlus™ semen sexing kit in hyperstimulated cows.

Materials and Methods

Estrous synchronization and follicular ablation

Cows (n= 17) were Angus and Angus crosses maintained on pasture. Ten cows were four to five weeks postpartum. On Day 0, cows were rectally palpated and given 25mg prostaglandin (IM; ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO). A CIDR (Eazi-Breed CIDR® device; DEC International, NZ) was inserted into those without corpora lutea (CL). On Day 3, cows were palpated, CIDRs were removed, and those with CLs were given 25mg prostaglandin. Estrous checks were performed every 12 hrs with the utilization of KAMAR® heat detection patches. On Day 6 following observed estrus, follicles greater than 5mm were destroyed via ultrasound-guided transvaginal ablation. CIDR devices were inserted into those cows with CLs smaller than 12mm.

Superovulation and Artificial Insemination

Forty-eight hours following follicular ablation, FSH treatment (Sioux Biochemical, Inc., Sioux Center, IA) treatment was initiated (IM; 20 mg total in a decreasing doses; BID) and continued for 4 days. CIDRs were removed 48 hrs into the FSH treatment and 25mg prostaglandin was administered concurrent with the fifth and sixth FSH injections. Cows were monitored for estrus every 6 hrs and 200μg GnRH (OvaCyst®; VedCo Inc., St. Joseph, MO) was administered at standing estrus. Artificial insemination was performed 12 hrs later.

Groups

Cows were randomly divided into four groups based upon time of estrus in a two-by-two factorial arrangement. Group 1 (C) was inseminated with 1ml frozen-thawed semen incubated at 37°C for 20 minutes. Group 2 (HP) was inseminated with 1ml of frozen-thawed, HeiferPlus™ treated semen, as per manufacturer instructions. Group 3 (Tryp+ HP) was inseminated with 1ml frozen-thawed semen rinsed using the trypsin/ percoll method (Appendix C), resuspended in semen extender (20ml egg yolk, 1.856g NaCitrate, 1g glucose, 80mls dH_2O, pH 7.0) and treated with HeiferPlus™. Group 4 (Tryp) was inseminated with 1ml frozen-thawed semen rinsed using the trypsin/ percoll method. All semen came from the same ejaculate of one bull.

Embryo Collection

Embryos were collected via non-surgical uterine lavage seven days post AI using one liter of 1x PBS solution containing 15mls BCS (HyClone, Logan, Utah), 50,000u Penicillin, and 50mg Streptomycin (Mediatech). The embryos were rinsed in ViGro Plus

holding media, graded, and transferred to individual 0.20ml PCR tubes in approximately 3µls holding media. Embryos were stored at -20°C until analysis.

PCR analysis

Embryos were allowed to thaw for five minutes at room temperature. Three microliters of Proteinase K (1µg/µl) solution were added to PCR tubes and tubes were incubated at 55°C for 10 minutes to digest zona pellucida. The tubes were placed in a 100°C water bath for 10 minutes to inactivate the Proteinase K and cooled to room temperature before the PCR reaction mixture was added. A multiplex PCR was performed using two sets of primers (Invitrogen, Carlsbad, CA). A bovine specific primer pair was used to amplify a 219 bp fragment from a bovine 1.715 satellite (forward: 5'- TGA GGC ATG GAA CTC CGC TT- 3'; reverse: 5'- GGT GGT TCC ACA TTC CGT AGG AC- 3'). A 131 bp Y-chromosome fragment was amplified (forward: 5' GAT TGT TGA TCC CAC AGA AGG CAA TC – 3'; reverse: 5' GAA CTT TCA AGC AGC TGA GGC ATT TA -3') (Xu, 2006). PCR mixture: 2.5µl Taq buffer (Promega, Madison, WI), 0.25u Taq DNA Polymerase (Eppendorf, Westbury, NY), 3µl $MgCl_2$ (Promega), 2µl dNTPs (Invitrogen), 9.75µl sterile H_2O, 0.25µl of each satellite primer and 2µl of each Y-primer (Invitrogen). DNA isolated from bovine testis and ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls.

PCR was initiated with a cycle of 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 64°C for 30 sec, extension at 72°C for 30 sec, and a final hold at 72°C for 10 min. Three microliters of 6x loading dye (Promega) were added to 12µl of PCR product and analyzed on 1.6% agarose gel

containing 3µl EtBr 1% (Fisher Scientific). One band at 219 bp denoted a female embryo while two bands, one at 219 bp and one at 131 bp, indicated a male embryo.

After analyzing 33.04% of the embryos (n= 37), a NCBI BLAST was performed on the male-specific primers and it was determined that the region of amplification was not male specific. An attempt was made to amplify a different segment of the Y chromosome from the previous PCR product.

PCR analysis

For those samples already subjected to PCR (n= 37), PCR product was thawed to room temperature and 5µl was used as template for a multiplex PCR using two sets of primers (Invitrogen, Carlsbad, CA). For the remaining embryos, PCR tubes containing embryos were allowed to warm to room temperature, 3µls Proteinase K (1µg/µl) was added, and the tubes were centrifuged for approximately 20 seconds. The tubes were placed in a thermocycler (Mastercycler® gradient, eppendorf) and were incubated at 55°C for 12 minutes to allow digestion of the zona pellucida, then heated to 98°C for 10 minutes to inactivate the Proteinase K, and held at 4°C. A segment of bovine β-actin (385 bp) was used as an internal positive control (forward: 5'- CCG AGG ACT TGA TTG TAC ATG G- 3'; reverse: 5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3'). A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCT TCC TTG TGC ACA GAC AG– 3'; reverse: 5' TTA TTG TGG CCC AGG CTT GT-3') (Daneau *et al*, 1995). Primers were diluted to 250pmoles/ µl in TE stock, diluted 1:10 in DepC H_2O working stock. PCR mixture: 10µl Go-taq (Promega, Madison, WI), 1µl of each β-actin primer and 2µl of each SRY primer. DNA isolated from bovine testis and

ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls and a negative control contained no DNA. PCR was initiated with an initial cycle of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec, hold at 72°C for 3 min, and a final hold at 4ºC.

Southern Blot

Southern blots were performed on each of the PCR products. Thirty microliters of double distilled H_2O was added to the PCR products, followed by 6µl 1N NaOH. The reaction was incubated at 37ºC for five minutes. Following denaturation, 20µl of 20x SSPE was added, followed by 200µl 6x SSPE. Using a vacuum-mediated dot blot manifold, samples were added to 6x SSPE pre-soaked nylon membrane (Biobond™-Plus Nylon Membrane; Sigma, St. Louis, MO) over two pieces of Whatman paper. Samples were run in duplicate, along with positive controls ranging from 2.0ng to 0.04ng DNA purified from PCR product (Qiagen, QIAquick® PCR purification kit, US). The membrane was crosslinked twice (Spectrolinker; Spectronics Corporation, Westbury, NY) at the 'optimal crosslink' setting. Membrane hybridization was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK) with overnight hybridization at 55ºC. CDP-Star™ chemiluminescent detection system was used with a 25 minute exposure on Kodak BioMax Light Film (Kodak, Cedex, France). Membranes were first probed for the SRY gene segment and then stripped by washing in boiling 6x SSPE and 0.5% SDS for 15min, twice. Membranes were then probed for the β-actin segment.

Results

Results from the Southern blot of PCR product supported the results of the original multiplex PCR amplification. A total of 112 embryos were collected from seventeen cows. Chi square analysis showed no difference among groups with regards to ovulations versus number of embryos produced. Single factor ANOVA showed no significant difference in the number of ovulations, embryos collected, or unfertilized oocytes (UFOs) collected between Control, HP, Tryp, or Tryp+ HP groups (Table 4.1).

TABLE 4.1. Comparison of Number of Ovulations, Embryos, and UFOs[1]

	Control (n= 6)	**HP (n= 6)**	**Tryp (n= 5)**	**Tryp+ HP (n= 5)**	**p-value**
Ovulations[2] (Mean ± SEM)	83 (13.83 ± 1.65)	70 (11.67 ± 1.26)	87 (17.46 ± 0.30)	71 (14.21 ± 1.27)	0.641
Embryos (Mean ± SEM)	30 (5.00 ± 1.40)	37 (6.17 ± 1.20	19 (3.85 ± 0.79)	26 (5.22 ± 1.49)	0.958
UFOs[1] (Mean ± SEM)	3 (0.50 ± 0.09)	9 (1.51 ± 0.23)	36 (7.24 ± 2.11)	12 (2.43 ± 1.07)	0.260

[1] UFOs: unfertilized oocytes collected.

[2] The number of ovulations was determined by the number of corpora lutea present at time of embryo collection.

TABLE 4.2. Sex Ratio of Embryos by Group as Determined by PCR/ Southern Blot

	Control (n= 30)	**HP (n= 37)**	**Tryp (n= 19)**	**Tryp+HP (n= 26)**	**p-value**
Number of Female (%)	19 (63.33)	17 (45.95)	15 (78.95)	13 (50.00)	0.08
Number of Male (%)	11 (36.67)	20 (54.05)	4 (21.05)	13 (50.00)	

Table 4.2 shows the sex of the embryos produced from cows of each group. Chi-Square analysis showed no significant difference in sex ratio of embryos collected from each group (Table 4.2). Interestingly, when the data is combined into Control (C and Tryp; n= 49) and HP (HP and Tryp+HP; n= 63), the Control group produced significantly more female embryos than the HP group (Table 4.3).

TABLE 4.3. Sex Ratio of Embryos by Merged Groups

	Control (n= 49)	**HP (n= 63)**	**p-value**
Number of Female (%)	34 (69.39)	30 (47.62)	0.021
Number of Male (%)	15 (30.61)	33 (52.38)	

Conclusions and Discussion

Results of this study showed that HeiferPlus^{TM} did not influence the sex ratio in favor of females in hyperstimulated cows, rather, the combined Control group produced a significantly higher proportion of female embryos than the combined HP group. Although unlikely, if HeiferPlus's mode of action works post-implantation, causing more male fetuses to abort resulting in a higher proportion of female offspring, its effects would not be observed in this study. Future studies should include the efficacy of HeiferPlus^{TM} on single-ovulating cows in which the fetuses can be carried to term. It would also be interesting to perform a semen analysis to detect changes in motility or progressive forward motility in HeiferPlus^{TM} treated sperm samples.

CHAPTER V

EXPERIMENT THREE: HEIFERPLUS™ EFFICACY IN SINGLE-OVULATING COWS

Objective

Two previous studies indicated that HeiferPlus™ semen sexing kit did not sway the sex ratio in favor of females in hyperstimulated cows (Chapters III and IV). The objective of this study was to evaluate the efficacy of HeiferPlus™ in single-ovulating cows synchronized via OvSynch®.

Materials and Methods

Ovulation synchronization and AI

Cows were Angus (n= 77) and Angus crosses (n= 75) maintained on pasture. Ovulation was synchronized using a modified OvSynch® protocol. On Day 0, cows (n= 152) were administered 200μg GnRH (IM; OvaCyst). On Day 7, cows were given 25mg prostaglandin, and on Day 9, given 100μg GnRH. Cows that exhibited signs of estrus before Day 9 were eliminated. Twelve hours after the second GnRH injection, cows were randomly divided into Control (n= 75) or HeiferPlus™ (HP; n= 77) groups. Controls were inseminated with 0.5mls frozen-thawed semen (semen volume determined by farm protocol) incubated at 37°C for 20 minutes and HPs were inseminated with 0.5mls of frozen-thawed HeiferPlus™ treated semen. Semen came from six different bulls and semen from each bull was used for an average of 25.3 inseminations, which a range of 17 to 37. Two AI technicians performed the inseminations.

Pregnancy Detection and Sex Determination

Pregnancy confirmation was performed by ultrasound with a 5.0- MHz probe (Aloka) at 36 to 38 days following AI. Visualization of a heartbeat confirmed pregnancy. Between 55 and 58 days of gestation, fetal sex was determined by ultrasound.

Semen Analysis

A computer-automated semen analyzer (CASA; Hamilton-Thorne Research, Beverly, MA) was used to evaluate semen from six of the eight bulls used in these studies (includes all experiments described; semen was unavailable from two bulls). Semen from each bull was used as a control as well as treated with HP as per manufacturer instructions. Following incubation, 10µl semen was diluted with 30µl bovine serum albumin 10% (Irvine Scientific, Santa Ana, CA). Samples were evaluated in duplicate for sperm concentration, motility, and progressive forward motility.

Results

Overview

Of the 152 cows inseminated, 51.3% (n= 78) were pregnant at Day 35. Of the cows inseminated with HP treated semen, 54.5% became pregnant, while 48.0% of the controls (n= 36) were pregnant. HP treated cows (n= 42) resulted in 54.8% male (23/ 42) and 45.2% female (19/ 42) fetuses. Control cows had 52.8% male (19/ 36) and 47.2% female (17/ 36) fetuses at 58 days gestation. Calving records showed that three Controls and one HP calf aborted after 58 days gestation, and one Control and one HP calf died within 2 weeks of birth. One Control cow and one HP cow were sold before calving, so calf sex

data is unavailable. The calf sex recorded on the farm records confirmed the ultrasound data. Chi square analysis showed no significant difference in the sex ratio between Control and HP groups.

Breed Effect

Pregnancy rates were similar for both breeds: of the 78 cows that were pregnant at Day 30, 44.9% were Angus and 55.1% were crosses. Of the total Angus Control group (n= 38), 47.4% became pregnant (18/ 38) and of the total Angus HP group , 43.6% became pregnant (17/ 39). Of the total crosses Control group, 48.6% became pregnant (18/ 37), whereas of the total crosses HP treated group, 65.8% became pregnant (25/ 38). Control Angus (n= 18) produced 55.6% females and HP Angus (n= 17) produced 47.1% females. Control crosses (n= 18) produced 38.9% females and HP crosses (n= 25) produced 44.0% females.

Bull Effect

Pregnancy rates among bulls were not different, with a mean pregnancy rate of 51.5%. Although some bulls produced a slightly higher ratio of females, the difference was not statistically significant.

Semen Analysis

Computer automated semen analysis measured sperm concentration, motility, and progressive forward motility. Single-factor ANOVA showed no significant difference in motility or progressive forward motility of Control versus HeiferPlus^{TM} treated semen in any of the bulls.

Conclusions and Discussion

Results of this study indicated that HeiferPlus™ semen sexing kit did not sway the sex ratio in favor of females in single ovulating cows synchronized via OvSynch®. These results support the evidence that HeiferPlus™ did not sway the sex ratio in favor of females in hyperstimulated cows. HeiferPlus™ treatment did not affect the number of embryos collected or the pregnancy rate. The semen analysis showed no significant difference in motility or progressive forward motility between Control and HeiferPlus™ treated semen.

CHAPTER VI

EXPERIMENT FOUR: HEIFERPLUS™ EFFICACY IN HYPERSTIMULATED COWS INSEMINATED 12 HRS POST FIRST OBSERVED STANDING ESTRUS

Objective

Because the breeding recommendations changed after Experiment One (Chapter III) and the overall number of embryos collected and analyzed from both Experiments One and Two was low, it was decided to repeat the trial with insemination at 12 hrs post first observed estrus. Also, there is evidence that the timing of insemination relative to ovulation may affect the sex ratio. The objective of this experiment was to determine the efficacy of HeiferPlus™ semen sexing kit in hyperstimulated cows inseminated 12 hrs following first observed standing estrus.

Materials and Methods

Estrous synchronization and follicle ablation

On Day 0, cows (n= 25) were rectally palpated and significant ovarian structures were recorded. They were given 25mg prostaglandin (IM; ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO). On Day 7, all animals were given 25mg prostaglandin and estrous checks were conducted every 12 hrs with the assistance of KAMAR® heat detection patches (Kamar, Inc. Steamboat Springs, CO). On Day 6 following observed estrus, follicles greater than 5mms were destroyed via ultrasound-guided transvaginal ablation, 25mg prostaglandin was administered, and those cows with corpa lutea less than 15mm received a CIDR device (Eazi-Breed CIDRs; DEC International, NZ).

Superovulation and Artificial Insemination

Forty-eight hours following follicular ablation, FSH treatment was initiated (IM; 20mg total in decreasing divided doses; BID) and continued for four days. CIDRs were removed 48 hrs into the FSH treatment and 25mg prostaglandin was administered concomitant with the fifth and sixth FSH injections. Estrous checks were conducted every six hours and cows were administered GnRH (OvaCyst) at first observed standing estrus. Cows were randomly assigned to Control (n= 12) or HeiferPlus™ (HP; n= 13) groups. Control animals were inseminated with 1ml frozen-thawed semen incubated at 37°C for 20 minutes while HP animals were inseminated using 1ml frozen-thawed, HeiferPlus™ treated semen, prepared as per manufacturer instructions (see Appendix B). All semen came from the same ejaculate of one bull.

Embryo Collection and Freezing

Embryos were collected via non-surgical uterine lavage seven days post AI using one liter of 1x PBS solution containing 15mls bovine calf serum (HyClone, Logan, Utah), 50,000u Penicillin, and 50mg Streptomycin (Mediatech, Inc., Herndon, VA). The embryos were graded, transferred to a 4-well plate containing holding media (ViGro Holding Plus; Bioniche), rinsed, and moved to individual 0.2ml PCR tubes in approximately 3µls holding media. Embryos were stored at ⁻80°C until analysis.

PCR

PCR tubes containing embryos were allowed to warm to room temperature, 3µls Proteinase K (1µg/µl) was added, and the tubes were centrifuged for approximately 20

seconds. The tubes were placed in a thermocycler (Mastercycler® gradient, eppendorf) and were incubated at 55°C for 12 minutes to allow digestion of the zona pellucida, heated to 98°C for 10 minutes to inactivate the Proteinase K, and held at 4°C. A multiplex PCR was performed using two sets of primers (Invitrogen, Carlsbad, CA). A segment of bovine β-actin (385 bp) was used as an internal positive control (forward: 5'-CCG AGG ACT TGA TTG TAC ATG G- 3'; reverse: 5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3'). A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCT TCC TTG TGC ACA GAC AG– 3'; reverse: 5' TTA TTG TGG CCC AGG CTT GT-3') (Daneau *et al*, 1995). Primers were diluted to 250pmoles/ µl in TE stock, then diluted 1:10 in DepC H_2O working stock. PCR mixture: 10µl Go-taq (Promega, Madison, WI), 1µl of each β-actin primer and 2µl of each SRY primer. DNA isolated from bovine testis and ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls and a negative control contained no DNA. PCR was initiated with a cycle of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec, hold at 72°C for 3 min, and then a final hold at 4ºC.

Southern Blot

Southern blots were performed on each of the PCR products. Thirty microliters of double distilled H_2O was added to the PCR products, followed by 6µl 1N NaOH. The reaction was incubated at 37ºC for five minutes. Following denaturation, 20µl of 20x SSPE was added, followed by 200µl 6x SSPE. Using a vacuum-mediated dot blot manifold, samples were added to 6x SSPE pre-soaked nylon membrane (Biobond™-Plus

Nylon Membrane; Sigma, St. Louis, MO) over two pieces of Whatman paper. Samples were run in duplicate, along with positive controls ranging from 2.0ng to 0.04ng DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, USA). The membrane was crosslinked twice (Spectrolinker, Spectronics Corporation, Westbury, NY) at the 'optimal crosslink' setting. Membrane hybridization was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK) with overnight hybridization at 55ºC. CDP-Star™ chemiluminescent detection system was used with a 25min exposure on Kodak BioMax Light Film (Kodak, Cedex, France). Membranes were first probed for the SRY gene segment and then stripped by washing in boiling 6x SSPE and 0.5% SDS for 15min, twice. Membranes were then probed for the β-actin segment.

Data Analysis

Embryos were divided into two groups: 'transferable' included viable embryos while 'degenerate' encompassed those embryos 8 cells or less or those that appeared to have deteriorated. 'High producing' cows were defined as those producing greater than five embryos, while 'low producing' cows produced less than or equal to five embryos. Chi square analysis was used to evaluate differences in proportions, whereas single-factor analysis of variance (ANOVA) was used to determine differences in means of embryos and unfertilized oocytes collected.

Results

A total of 113 embryos were collected from 24 cows. Two Control cows were omitted from the study due to failed hyperstimulation (≤ 2 CLs at time of embryo collection).

Embryo sex was determined for 96.46% (n= 109) of the samples (four Control samples were lost due to PCR tubes breaking).

Table 6.1. Comparison of Embryos and UFOs[1]

	Control (n= 11)	**HP (n= 13)**	**p-value**
Total Embryos (Mean ± SEM)	38 (3.45 ± 0.32)	75 (5.77 ± 0.31)	0.151
Total UFOs[1] **(Mean ± SEM)**	58 (5.27 ± 0.72)	68 (5.23 ± 0.42)	0.929

[1] UFOs: unfertilized oocytes collected.

Table 6.1 shows the total number of embryos and UFOs collected for each group, as well as means and standard error for the means. Single factor ANOVA showed no significant difference between embryos and UFOs collected for each group. Surprisingly, the Control group produced a significantly higher proportion of females (Table 6.2) than the HP group (58.8% vs. 37.5%, respectively).

Table 6.2. Sex Ratio of Embryos by Group as Determined by PCR/ Southern Blot

	Control (n= 34)	**HP (n= 75)**	**p-value**
Number Female (%)	20 (58.8)	28 (37.3)	0.036
Number Male (%)	14 (41.2)	47 (62.7)	

There were no differences between high embryo producing and low embryo producing cows with regards to sex ratio, nor were there differences in transferable and degenerate embryo sex ratio by group.

Conclusions and Discussion

As in the previous studies, the Control group produced a higher proportion of female embryos than the HeiferPlus^{TM} group, indicating that HeiferPlus^{TM} did not sway the sex ratio in favor of females. Future studies will address the effect of semen incubation on the sex ratio (Appendix A). There were no differences between high embryo producing and low embryo producing cows with regards to sex ratio, nor were there differences in transferable and degenerate embryo sex ratio by group

The data pertaining to the number of corpora lutea present at the time of embryo collection was not available for this study. In this experiment, the PCR tubes containing the embryos were stored at ¯80ºC. After the samples thawed to room temperature, the tubes were brittle and four tubes cracked in the centrifuge.

CHAPTER VII

POOLED DATA FROM HYPERSTIMULATED COWS

Objective

In total, three trials were conducted to examine the efficacy of HeiferPlus^{TM} semen sexing kit in hyperstimulated cows (Chapters III, IV, and VI). In this assessment, the data were pooled to increase the power of the statistical analysis.

Materials and Methods

The following results encompass the data collected from Experiments One, Two, and Four. The results of Experiment Three, HeiferPlus^{TM} Efficacy in Single Ovulating Cows, were not included. Overall, 271 embryos were collected from superovulated cows used in these studies and a sex was assigned to 265 of them. A total of 104 embryos came from Control cows and 167 embryos came from HeiferPlus^{TM} (HP) treated cows.

Results

Chi square analysis showed that the Control group produced a significantly higher proportion of female embryos than the HP group ($p< 0.0005$). Control cows produced 65.0% female embryos, while HP cows produced 43.0% female embryos (Table 7.1).

Table 7.1. Sex Ratio of Embryos by Group as Determined by PCR/ Southern Blot

	Control (n= 100)	**HP (n= 165)**	**p-value**
Number of Female (%)	65 (65.0)	71 (43.0)	0.0005
Number of Male (%)	35 (35.0)	94 (57.0)	

Table 7.2. Comparison of Ovulations, Embryos and UFOs[1] between Groups

	Control (n= 29)	HP (n= 34)	p-value
Total Ovulations[2] (Mean ± SEM)	371 (17.67 ± 0.46)	412 (16.48 ± 0.33)	0.656
Total Embryos (Mean ± SEM)	104 (3.59 ± 0.16)	167 (4.91 ± 0.15)	0.284
Total UFOs[1] (Mean ± SEM)	111 (3.83 ± 0.23)	134 (3.94 ± 0.15)	0.709

[1] UFOs: unfertilized oocytes collected.

[2] The number of ovulations was determined by the number of corpora lutea present at time of embryo collection. Ovulation data was unavailable for 17 cows used in these studies and was not included in the analysis.

Single factor ANOVA showed no difference in number of ovulations, embryos, or UFOs collected between groups. Of the two bulls used in the superovulation studies, one bull produced an overall higher proportion of females ($p < 0.05$). There were no differences in embryo grade in Control versus HP embryos.

The sequences were subcloned into plasmids and confirmed by Clemson University Genomics Institute (CUGI) sequencing group.

Conclusions and Discussion

Although there was debate over whether the original male-specific primers used in this study were strictly male-specific, the positive controls worked in each sample set that was analyzed, giving credence to those primers. When a sub-sample of the PCR product was used as template for a second multiplex PCR reaction using novel primer pairs, subsequent Southern blot results confirmed the original assigned embryo sex.

The pooled data verified the pre-existing analyses: HeiferPlus^{TM} did not sway the bovine sex ratio in favor of females. It also supported the observation that the Control group produced significantly more females than the HP group. Because one bull produced an significantly higher proportion of female embryos, there may be a bull effect, although the difference was not dependent upon treatment. Future studies will test the hypothesis that a twenty minute semen incubation at 37°C sways the sex ratio in favor of females (Appendix A).

CHAPTER VIII

USE OF A COMBINED PCR/ SOUTHERN BLOT ASSAY FOR SEXING BOVINE EMBRYOS

Introduction

A reliable, sensitive, and expeditious method of genetic analysis is necessary to detect specific DNA sequences in large quantities of samples with limited template. The most widely utilized method of gene amplification, polymerase chain reaction (PCR), imparts inconsistent results when assessing embryos and other samples with limited template, especially when copies of target genes are unequal. The object of this study was to evaluate the efficacy of Southern blot analysis combined with PCR multiplex amplification.

Materials and Methods

Embryos

Hyperstimulated cows were artificially inseminated 12 hrs following estrus and concomitant 200µg GnRH (IM; OvaCyst®; VedCo Inc., St. Joseph, MO). Embryos were non-surgically collected seven days post-insemination and were graded using the International Embryo Transfer Society grading system (Manual of the International Embryo Transfer Society, 3rd Edition). Developmental stages spanned from a four-cell to the blastocyst. The embryos were transferred to a 4-well plate containing holding media (ViGro Holding Plus; Bioniche Animal Health, Inc.), rinsed, and placed in individual PCR tubes (0.2ml) with approximately 3µls holding media. Samples were stored at ¯20°C until analysis.

PCR

PCR tubes containing embryos were allowed to warm to room temperature and 3µl Proteinase K (1µg/µl) was added. The tubes were placed in a thermocycler (Mastercycler® gradient, Eppendorf) and were incubated at 55°C for 12 minutes to allow digestion of the zona pellucida. They were heated to 98°C for 10 minutes to inactivate the Proteinase K, and then held at 4°C. A multiplex PCR was performed using two sets of primers (Invitrogen, Carlsbad, CA). A segment of bovine β-actin (385 bp) was used as an internal positive control (forward: 5'- CCG AGG ACT TGA TTG TAC ATG G- 3'; reverse: 5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3'). A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCT TCC TTG TGC ACA GAC AG– 3'; reverse: 5' TTA TTG TGG CCC AGG CTT GT-3') (Daneau *et al*, 1995). Primers were diluted to 250pmoles/ µl in TE stock and then diluted 1:10 in DepC water for a working stock. PCR mixture: 10µl Go-taq (Promega, Madison, WI), 1µl of each β-actin primer and 2µl of each SRY primer. DNA isolated from bovine testis and ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls. PCR was initiated with a cycle of 95°C for 3 min followed by 35 cycles of denaturation 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 4ºC.

For agarose gel analysis, 5µl of PCR product was added to 3µl of loading dye (30% glycerol, bromophenol blue) and analyzed on 1.6% agarose gel containing 3µl ethidium bromide 1% (Fisher Scientific). One band at 385 bp denoted a female embryo while two

bands, one at 385 bp and one at 532 bp, indicated a male embryo. Lanes with no visible bands were considered to be inconclusive.

Southern Blot

Dot blots were performed on each of the remaining PCR products (15µl). Thirty microliters of double distilled H_2O was added to PCR products, followed by 6µl 1N NaOH. The reaction was incubated at 37ºC for five minutes. Subsequent to denaturation, 20µl of 20x SSPE was added, followed by 200µl 6x SSPE. Using a dot blot manifold, samples were added to 6x SSPE pre-soaked nylon membrane (Biobond™-Plus Nylon Membrane; Sigma, St. Louis, MO) over two pieces of Whatman paper. Samples were run in duplicate, along with positive controls ranging from 2.0ng to 0.04ng DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, Valencia, CA). The membrane was crosslinked twice (Spectrolinker, Spectronics Corporation, Westbury, NY) at the 'optimal crosslink' setting. Membrane hybridization was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK) with overnight hybridization at 55ºC. CDP-Star™ chemiluminescent detection system was used with a 20min exposure on Kodak BioMax Light Film (Kodak, Cedex, France). Membranes were first probed for the SRY gene segment and then stripped by washing in boiling 6x SSPE/ 0.5% SDS for 15min, twice. Membranes were then probed for the β-actin segment.

Results

Chi square analysis showed that PCR and Southern blot analysis used in tandem were more sensitive and reliable ($p < 0.05$) than ethidium bromide staining of PCR product

alone and allowed for high-throughput examination of samples with low DNA copy numbers (Table 8.1). PCR analysis showed that 37.6% of embryos were male (n= 70), 39.8% were female (n= 74), and 22.6% (n= 42) showed inconclusive results (Figure 8.1). The same samples were subjected to dot blot analysis and revealed 48.4% male (n= 90) and 51.6% female (n= 96) embryos, with no inconclusive samples (Figures 8.2 and 8.3). Of the inconclusive PCR products, 38.1% were male (n= 16) and 61.9% were female (n= 26). Interestingly, Southern blot analysis revealed six samples that contradicted the gel analysis, representing 3.2% of the total samples evaluated: one female that the gel demonstrated as male and five males that the gel indicated were females.

TABLE 8.1. Comparison of PCR versus PCR Followed by Southern Blot

	No. Male (%)	**No. Female (%)**	**No. Inconclusive (%)**
PCR	70 (37.6)	74 (39.8)	42 (22.6)
PCR and Southern blot	90 (48.4)	96 (51.6)	0

Figure 8.1. Agarose Gel Following Multiplex PCR Reaction

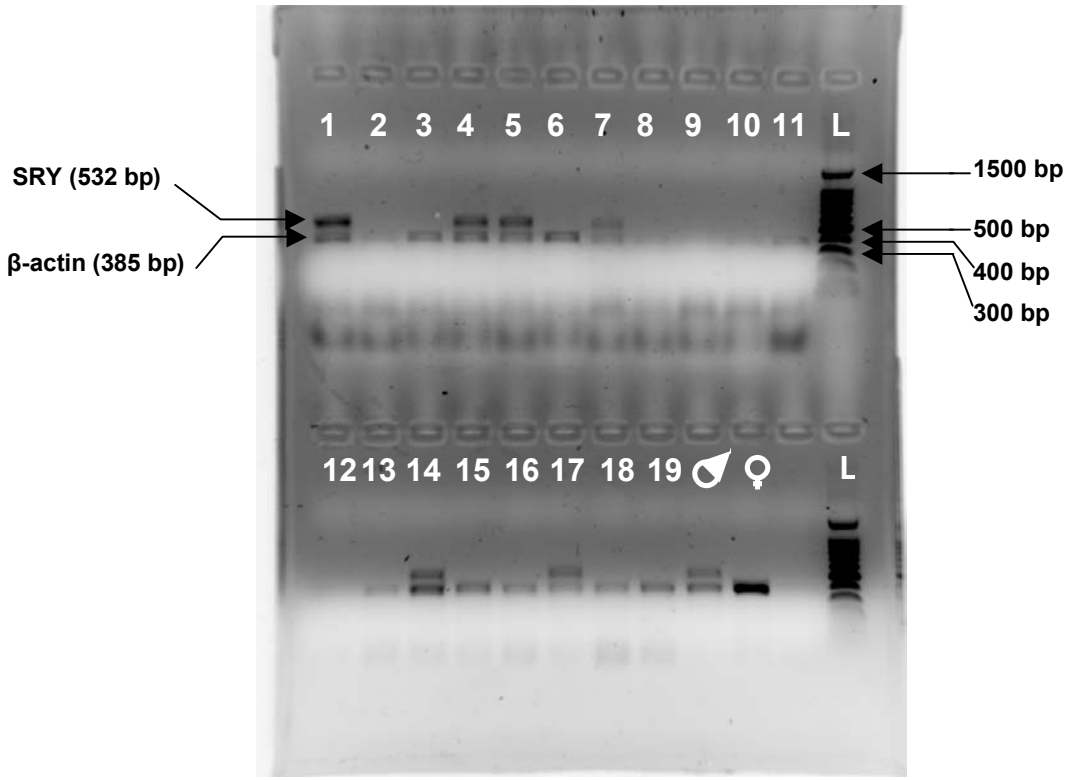


Figure 8.1 shows a picture of 1.6% Agarose gel stained with ethidium bromide. The lanes with one band at 385 bp indicate a female embryo, while lanes with two bands, one at 385 bp and one at 532 bp, denote a male embryo. On this gel, many samples showed inconclusive results (Lanes 8, 9, 10, 12). The male, female, and negative controls are labeled in the bottom right corner. The 100 kb ladder is in the lanes on the right and is labeled 'L'.

Figure 8.2. Results of Southern Blot Following Male- Specific Probe

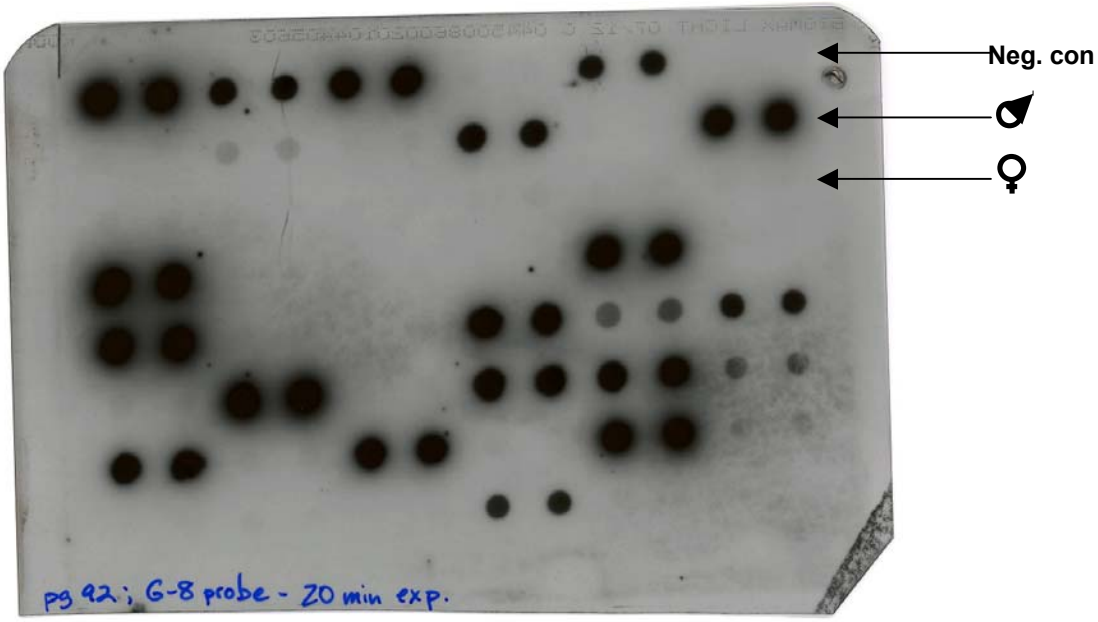


Figure 8.2 is a picture of an X-ray film showing the results of a Southern blot after probing for the male- specific segment. The dark spots represent positive samples, indicating a male embryo. All samples were blotted in duplicate. The negative control and the male and female controls can be found in the upper right corner.

Figure 8.3. Results of Southern Blot Following β-actin Probe

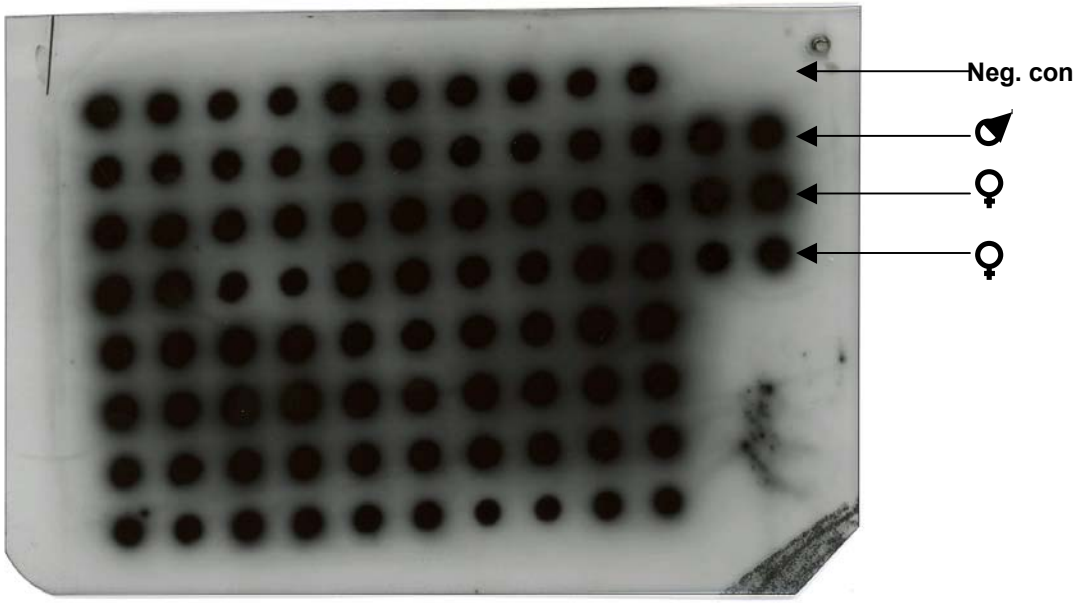


Figure 8.3 is a picture of an X-ray film showing the results of the same blot (Figure 8.2) following membrane stripping and subsequent probing for the β-actin product. The dark spots indicate the presence of β-actin, which can be found in all cells of the embryo and therefore acts as a positive control. All samples were positive for β-actin and all were blotted in duplicate. The negative control and the male and female controls can be found in the upper right corner.

Conclusions and Discussion

This study demonstrated that PCR amplification followed by Southern blot analysis provided accurate, sensitive, and consistent results in the evaluation of samples with low DNA concentrations. A 2006 study by Shekhar *et al* compared a Southern dot blot technique to PCR for detection of white spot syndrome virus in shrimp tissues. Each technique was used independently and researchers found that PCR analysis was more sensitive than Southern blotting of crude DNA extracts, although the blots were able to

detect the presence of genes in some tissues (eye and eye stalk) that PCR could not. In a clinical study, Schiffman *et al* (1991) also compared Southern blot to PCR in order to identify human papillomavirus DNA and found that PCR detected more positive samples than Southern blot.

Boerner *et al* (1999) combined PCR with Southern blot by amplifying bovine herpesvirus 4 (BHV-4) from experimentally infected calves. Using PCR alone, they detected a lower limit of 10fg of BHV-4, whereas by blotting PCR product, they were able to detect down to 1fg: a ten-fold increase in sensitivity. Results indicate that blotting PCR product provides more sensitive analysis than PCR analysis or Southern blotting of crude DNA alone.

CHAPTER IX

OVERALL CONCLUSIONS, DISCUSSION, AND FUTURE WORK

Results from these experiments indicate that HeiferPlus™ did not sway the sex ratio in favor of female calves in hyperstimulated cows or single-ovulating cows synchronized via OvSynch®. In the hyperstimulated cows, the Control group produced a significantly higher proportion of female embryos than the HeiferPlus™ group and, in single-ovulating cows, the Control group also produced a slightly higher proportion of females, although the difference was not significant. In the studies using hyperstimulated cows, no differences in embryo production (>5 or ≤5) or embryo grade (transferable versus degenerate) were observed.

HeiferPlus™ was inefficient at altering the sex ratio and, because the compound ingredients are unknown, it is difficult to attempt an explanation of its mode of action. A semen analysis was performed and no differences in Control or HeiferPlus™ motility or progressive forward motility were detected. If, as the manufacturer claims, HeiferPlus™ 'speeds up' X- bearing sperm and 'slows down' Y- bearing sperm at equal rates, it would be impossible to detect a difference in overall motility in a mixed X and Y sample. It would be interesting to analyze sexed semen with and without HeiferPlus™ treatment to evaluate differences in motility.

In Experiments One and Two, a subsequent NCBI BLAST reported that the amplified 'male-specific' region was found in female cow liver cDNA. To argue the validity of the primers, both the male and female positive controls in this study showed appropriate

bands in the agarose gel. Also, when the new primer set was used with PCR product to amplify new male- specific regions, the results confirmed the previous findings.

Although the trypsin/ percoll wash did not help to alter the sex ratio in Experiment 2, a study by Watkins *et al* (1996) found that when a human semen sample was centrifuged through an eight layer Percoll gradient, the lower fraction (X- bearing sperm) had higher motility, which it maintained over a 24 hr incubation period. This study could be applied to cows combined with a semen incubation period, in which a hyperstimulated cow would be inseminated with the lower fraction of sperm cells after an extended incubation.

Currently, it is not possible to alter the bovine sex ratio in a commercially feasible manner. Flow cytometry is expensive and other methods, such as albumin gradients, sex-specific surface proteins, and timing of insemination relative to ovulation, have conflicting reports of accuracy. Because it is difficult to exploit the only known difference between X- and Y- bearing sperm (mass), perhaps the sex of the embryo can be manipulated by the maternal side, through oviductal or ovarian influences. Further research is necessary to determine what establishes natural sex selection.

APPENDICES

Appendix A

Effect of Semen Incubation on Bovine Embryo Sex Ratio

Introduction

Swaying the sex ratio is beneficial in both agricultural and human fertility treatments. In the dairy industry, female calves are significantly more valuable than male calves because of their ability to produce milk and offspring, whereas in the beef industry, male calves are more desirable due to their rapid growth and efficient meat production. Although flow cytometers successfully sort X- and Y-bearing sperm, the method is expensive, requires highly trained personnel, and results in decreased sperm motility. It also decreases the amount of sperm cells per dose of semen, which may lead to decreased pregnancy rates. To date, there is no inexpensive, effective technique for manipulating the sex of offspring. Recent evidence suggests that the incubation of bull semen may sway the sex ratio in favor of female calves (Chapter 7). This study aimed to further evaluate the effect of semen incubation on the sex ratio of bovine embryos.

Materials and Methods

Estrous synchronization and follicular ablation

On Day 0, cows were rectally palpated and were administered 25mg prostaglandin (IM; ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO). On Day 7, all animals were given 25mg prostaglandin and estrous checks were conducted every 12 hrs with the utilization of KAMAR® heat detection patches (Kamar, Inc. Steamboat Springs, CO). Six days following observed estrus, follicles greater than 5mm were destroyed via

ultrasound-guided transvaginal ablation, 25mg prostaglandin was administered, and CIDRs were inserted (Eazi- Breed CIDR; DEC International, NZ).

Superovulation and Artificial Insemination

FSH treatment (Sioux Biochemical, Inc., Sioux Center, IA) was initiated 48 hrs following follicular ablation. Cows were treated with a total of 10mg FSH (IM; decreasing doses over 4 days; BID). CIDRs were removed 48 hrs into the FSH treatment. Estrous checks were conducted every six hours and 200µg GnRH (im; Cistorellin; Merial, Athens, GA) was administered at standing estrus. Cows were randomly assigned to Control (n= 8) or Treatment (n= 7) groups. Control animals were inseminated as per standard AI procedure with 1ml frozen-thawed semen, while Treatment animals were inseminated using 1ml of frozen-thawed semen allowed to incubate at 37°C for twenty minutes. All semen came from the same ejaculate of one bull (Bonview; Select Sires, Plain City, OH).

Embryo Collection and Freezing

Embryos were collected via non-surgical uterine lavage seven days post AI using one liter of 1x PBS solution containing 15mls bovine calf serum (HyClone, Logan, Utah), 50,000u Penicillin, and 50mg Streptomycin (Mediatech, Inc., Herndon, VA). The embryos were graded via the International Embryo Transfer Society grading system (Manual of the International Embryo Transfer Society, 3rd Edition), transferred to a 4-well plate containing holding media (ViGro Holding Plus; Bioniche), and rinsed. They were then placed in individual 0.2ml PCR tubes with approximately 3µls holding media. Embryos were stored at ¯20°C until analysis.

PCR

PCR tubes containing embryos were allowed to warm to room temperature and 3µls Proteinase K (1µg/µl) was added. The tubes were placed in a thermocycler (Mastercycler® gradient, Eppendorf) and were incubated at 55°C for 12 minutes to allow digestion of the zona pellucida. They were then heated to 98°C for 10 minutes to inactivate the Proteinase K, and held at 4°C. A multiplex PCR was performed using two sets of primers (Invitrogen, Carlsbad, CA). A segment of bovine β-actin (385 bp) was used as an internal positive control (forward: 5'- CCG AGG ACT TGA TTG TAC ATG G- 3'; reverse: 5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3') A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCT TCC TTG TGC ACA GAC AG– 3'; reverse: 5' TTA TTG TGG CCC AGG CTT GT-3') (Daneau *et al*, 1995). Primers were diluted to 250pmoles/ µl in TE stock and then diluted 1:10 in DepC water working stock. PCR mixture: 10µl Go-taq (Promega, Madison, WI), 1µl of each β-actin primer and 2µl of each SRY primer. DNA isolated from bovine testis and bovine ovary by Wizard® Genomic DNA Purification Kit (Promega) was used as positive controls and a negative control containing no DNA was run. PCR was initiated with a cycle of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec, hold at 72°C for 3 min, and then a final hold at 4ºC.

Southern Blot

Southern blots were performed on the PCR products. Thirty microliters of double distilled H_2O was added to PCR products, followed by 6µl 1N NaOH. The reaction was

incubated at 37ºC for five minutes. Subsequent to denaturation, 20µl of 20x SSPE was added, followed by 200µl 6x SSPE. Using a dot blot manifold, samples were added to 6x SSPE pre-soaked nylon membrane (Biobond^{TM}-Plus Nylon Membrane; Sigma, St. Louis, MO) over two pieces of Whatman paper. Samples were run in duplicate, along with positive controls ranging from 2.0ng to 0.04ng DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, Valencia, CA). The membrane was crosslinked twice (Spectrolinker; Spectronics Corporation, Westbury, NY) at the 'optimal crosslink' setting. Membrane hybridization was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System^{TM} (GE Healthcare, Buckinghamshire, UK) with overnight hybridization at 55ºC. CDP-Star^{TM} chemiluminescent detection system was used with a 20min exposure on Kodak BioMax Light Film (Kodak, Cedex, France).

Membranes were first probed for the SRY gene segment and then stripped by washing in boiling 6x SSPE and 0.5% SDS for 15min, twice. Membranes were then probed for the β-actin segment.

Results

Table A.1. Comparison of Ovulations, Embryos and UFOs[1] Between Groups

	Control (n= 8)	**Treatment (n= 7)**	**p-value**
Total Ovulations[2] (Mean ± SEM)	128 (16.0 ± 1.02)	100 (14.3 ± 1.07)	0.680
Total Embryos (Mean ± SEM)	91 (11.4 ± 0.55)	55 (7.9 ± 0.98)	0.251
Total UFOs[1] (Mean ± SEM)	13 (1.6 ± 0.24)	21 (3.0 ± 0.31)	0.214

[1] UFOs: unfertilized oocytes collected.

[2] The number of ovulations was determined by the number of corpora lutea present at time of embryo collection.

Table A.1 shows the total number of ovulations, embryos collected, and UFOs for each group, as well as means and standard error for the means. Single factor ANOVA showed no significant differences in the number of ovulations, embryos collected, or UFOs collected. Chi square analysis was used to determine if the proportion of ovulations versus embryos collected was affected by incubation and if incubation swayed the sex ratio. For both, there was no significant difference at the 0.05 level. The Control group produced 43 (47.25%) female and 48 (52.75%) male while the Treatment group produced 29 (52.73%) female and 26 (47.27%) male. Chi square analysis showed this was not significant ($p= 0.521$).

Conclusions and Discussion

Results of this study indicated that semen incubation at 37ºC for twenty minutes did not affect the quality of the semen, as determined by the proportion of embryos per ovulations collected, nor the sex ratio of the embryos produced from hyperstimulated cows. These results did not support previous studies that suggested that semen incubation may sway the sex ratio in favor of females.

A study of *in vitro* produced bovine embryos showed that a 24 hr semen incubation period resulted in significantly more female hatched blastocysts when compared with 0 or 6 hr semen incubation periods (Lechniak, 2003). Although the mode of action was unknown, Watkins *et al* (1996) found that X-bearing sperm had a significantly higher

percentage of motility, rapid progression, and hyperactiviation after 24 hr incubation when compared to Y-bearing sperm. They also had faster curvilinear and straight line velocities.

Future studies could include evaluation of the effects various durations of semen incubation on the sex ratio, with extended incubations of up to 24 hrs, as well as variations in incubation temperature could be tested. Also, it would be interesting to examine semen parameters of known X- and Y-sorted sperm samples before and after extended incubations to detect changes in motility or progressive forward motility of each group.

Appendix B

HeiferPlus™ Package Insert

HEIFER*plus*™

Indications: For production of *heifer* calves in dairy and beef cattle.

Dose: One unit/vial. Each unit will treat a single dose of semen (0.5 ml or 0.25 ml).

Storage: Store in freezer (-20°C) until use. Avoid exposure to direct sunlight.

Description: HEIFER*plus*™ is a spermagenic agent for sexing a single dose of bull semen. Each dose is lyophilized to maintain potency during storage. The agent is rehydrated by adding semen directly to the HEIFER*plus*™ vial. After a short incubation, the enriched semen is ready for insemination.

Mode of Action: Gender is determined by the sperm. HEIFER*plus*™ works by selectively "knocking out" the motility of only the Y-bearing (male) sperm. The treated sperm are sorted in the reproductive tract of the dam. The results are more ova fertilized by the X-bearing sperm (female). The gender of calves is enriched to an average of 75% female.

Instructions for use:

1. Warm the HEIFER*plus*™ vial to 98.6-100°F(37-38°C) in a water bath, tube warmer or incubator for a few minutes (to prevent cold shock).
2. Puncture a hole in the top septum of the HEIFER*plus*™ vial with a 14G needle or similar device.
3. Thaw semen as usual.
4. Cut the end of the semen straw as usual.
5. Insert the cut-end of the semen straw through the puncture in the vial septum.
6. To add the semen to the vial, grasp both the vial and straw in the palm of the hand and shake downward 3 or 4 times (similar to shaking a glass thermometer). Be certain all semen is in the vial.
7. Mix semen with contents of vial.
8. Transfer the enriched semen from the vial back into the straw. Do this by grasping the vial and straw in an inverted position and again shaking downward 3-4 times. Be certain all semen is in the straw.
9. **Incubate enriched semen at 98.6-100°F(37-38°C) for 20-30 minutes.**
10. Load straw into insemination gun and inseminate as usual.

Note: Incubation can be done in a thawing unit with the semen remaining in the straw. It is recommended to keep the vial attached to the straw. This will protect the a sperm from exposure to air and water. It will also assist in identification.

Insemination: The timing of insemination will increase the effectiveness of this product.

For natural heat: Breed **12 hours** following standing heat.

For synchronized AI: For best results, use an OVSYNCH® program incorporating GnRH (Cystorelin, Factrel, or Fertagyl) and prostaglandin (Lutalyse or Estrumate). Administer GnRH 48 hr. following the prostaglandin injection. Consult your veterinarian, if necessary.

- When using **OVSYNCH®**, *breed **12 hr.** following GnRH injection.*
- When using **Lutalyse or Estrumate only**, *breed **12 hr.** following standing heat or if by appointment, 72 hours following prostaglandin injection.*
- If using **PRID, Synchromate B, or CIDR**, *breed **12 hr.** following standing heat.*

For embryo transfer: When using HEIFER*plus*™ with superovulated donors, *the first breeding should be done **12 hr.** following standing heat. A second breeding is recommended 12 hr. following the first insemination.*

For *bull or steer* production, use Product No. BP025, BULL*plus*™.

Manufactured and distributed by: Emlab Genetics
Arcola, IL 61910 USA Phone:217-268-3324 Fax:217-268-4478
REVISED: 19.01.06

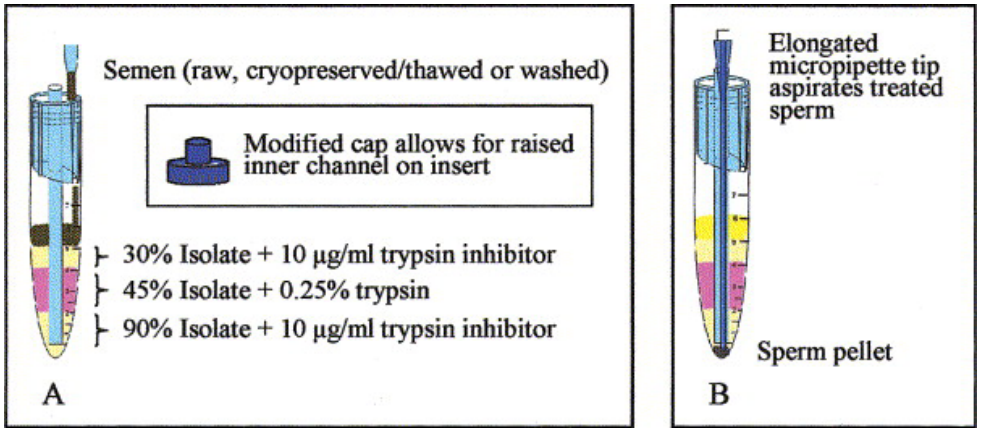
Appendix C

Trypsin/ Percoll Semen Wash Protocol

1. Pipette 2ml of 90% Percoll into receptacle
2. Pipette 2ml of 45% Trypsin LE into receptacle
3. Warm upright tube to 37ºC
4. Add semen by cutting off both ends of straw and allowing it to drain into receptacle (Figure C.1)
5. Cap and centrifuge at 1400 x g for 30 minutes
6. Place tom cat catheter on end of pipette to pick up sperm pellet without aspirating solution (Figure C.1)
7. Resuspend sperm pellet in semen extender to bring to a total volume of 1ml
8. Divide sample (sperm plus extender) into (2) 0.5ml straws
9. Inseminate as normal

-For Tryp+ HP group, follow HeiferPlus™ treatment instructions

Figure C.1. Trypsin/ Percoll Gradient Before (A) and After (B) Centrifugation



Specialized polypropylene (U.S. Food and Drug Administration medical grade) insert (Diversified Plastics Inc., Minneapolis, MN) for standard 15-mL polystyrene conical tube (Falcon 352095, BD Biosciences, Bedford, MA) facilitates the layering of multiple density gradients (A) and allows access to the pellet without contaminating the treated sperm (B).

Loskutoff. Semen washing using silica and trypsin. Fertil Steril 2005.

Note: The 30% Percoll layer was excluded from the current study.

Appendix D

Society for the Study of Reproduction Abstact 2006

Efficacy Determination of HEIFERPlus™ Semen Sexing Kit in Superovulated Cows

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Manipulating the sex ratio is beneficial in the dairy industry because female calves are significantly more valuable than males. In the beef industry, male calves are more desirable due to their rapid growth and efficient meat production. At present, the only proven methods of semen sexing (flow cytometry) are expensive and require fresh samples. The object of this study was to assess the efficacy of a commercially available kit, HEIFERPlus™ (Emlab Genetics, Arcola, IL), which uses frozen-thawed semen samples to skew the sex ratio in favor of females. This protocol requires 0.25cc thawed semen to be added to the HEIFERPlus™ vial, transferred back into the straw, and then incubated at 37°C for twenty minutes. Superovulated cows were artificially inseminated with HEIFERPlus™ treated semen (n= 6) or without treatments (controls; n= 5) and embryos were collected seven days post-insemination. Polymerase chain reaction (PCR) was performed on the embryos to amplify a fragment of the Y-chromosome (131 bp), as well as an internal control 1.715 satellite marker common to both sexes (219 bp). Bovine ovary and testis were used as positive control tissues. The satellite marker was detected in all embryos assayed. One-way ANOVA analysis showed no significant difference (P>

0.1) in the proportion of female to male morula and blastocyst stage embryos of treated cows versus control cows. The treated group produced 52.9% female embryos while the control group showed 64.7% females. Interestingly, no degenerate embryos (8-32 cells) were collected from the control group while degenerate embryos collected from the treated cows were 40% female. In conclusion, results indicate that HEIFERPlus^{TM} does not effectively influence the sex ratio in favor of female embryos in superovulated cows. Further research is necessary to evaluate the effects of HEIFERPlus^{TM} on the sex ratio of embryos produced by single ovulating cows inseminated relative to ovulation or by *in vitro* fertilization, as well as its influence on sperm quality, motility and fertilization.

Appendix E

Society for the Study of Reproduction Abstract 2007

High-Throughput Genetic Characterization of Bovine *In Vivo* Produced Embryos

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A reliable, sensitive, and expeditious method of genetic analysis is necessary to detect specific DNA sequences in large quantities of samples with limited template. The most widely utilized method of gene amplification, polymerase chain reaction (PCR), imparts inconsistent results when assessing embryos and other samples with limited template, especially when copies of target genes are unequal. The object of this study was to evaluate the efficacy of Southern Blot analysis following PCR multiplex amplification. Superovulated cows (n= 39) were artificially inseminated and embryos (n= 221) were non-surgically collected seven days post-insemination. Embryo development spanned from the four-cell stage to the blastocyst. PCR was performed on the embryos to amplify a segment of the Y-chromosome (532 bp) to identify males, in addition to a fragment of beta actin as an internal control (385 bp). Bovine ovary and testis DNA were used as positive controls. Dot blots of the PCR products were probed in duplicate for each gene segment (Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare) with controls ranging from 2.00 to 0.08 ng DNA. Results showed that 100.0% of the samples exhibited either the male specific sequence (n= 109) or both the male sequence

and beta actin sequence (n= 221). For forty of the embryos assessed, PCR product was analyzed on 1.6% agarose gel stained with ethidium bromide. Of these, 30.0% of embryos were male (n= 12), 42.5% were female (n= 17), and 27.5% (n= 11) showed inconclusive results. The same samples were subjected to dot blot analysis and revealed 40.0% male (n= 16) and 60.0% female (n= 24) embryos. Of the inconclusive PCR products, 18.2% were male (n= 2) and 81.8% were female (n= 9). Southern blot analysis revealed four embryos that contradicted the gel analysis, representing 13.8% of the samples evaluated with both methods: one female that the gel demonstrated as male and three males that the gel indicated were females. These methods used in tandem were more sensitive and reliable ($p < 0.01$) than ethidium bromide staining of PCR product alone and allowed for high-throughput examination of samples with low DNA copy numbers. This study demonstrated that PCR amplification followed by Southern Blot analysis provided accurate, sensitive, and consistent results in the evaluation of samples with low DNA concentrations. Further applications include analysis of embryo biopsies to genetically characterize embryos prior to transfer or freezing.

Appendix F

PCR Primers Utilized in Final Experiments

Table A.2. PCR P PCR Primers Utilized in Final Experiments

Name	Product Length	Forward Primer	Reverse Primer
β-actin	385 bp	5'- CCG AGG ACT TGA TTG TAC ATG G- 3'	5'- ACT GGT CTC AAG TCA GTG TAC AGG- 3'
G-8	532 bp	5'- TCT TCC TTG TGC ACA GAC AG- 3'	5'- TTA TTG TGG CCC AGG CTT GT-3'

(Daneau *et al*,. 1995)

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