

COMPARING CALF SEX RATIO AND SEMEN SEX RATIO DETERMINED BY
CONVENTIONAL PCR

A Thesis

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ABSTRACT

Thirty-nine ejaculates from 8 Holstein bulls were collected. Straws of semen (n=750) were distributed among dairies in three states. Ten straws per ejaculate were sent to Louisiana State University (LSU) Dairy Improvement Center for conventional polymerase chain reaction (PCR) analysis. Spermatozoal DNA was extracted and PCR analysis was done using one primer set amplifying a single copy 125 base pair (bp) section of the *Bos taurus* factor IX (Christmas factor) precursor (found on the X chromosome) and another primer set amplifying a single copy section of the *Bos taurus* sex determining region Y protein (SRY) gene (found on the Y chromosome). A 294 bp product from the *Bos taurus* glyceraldehydes-phosphate-dehydrogenase (GAPDH) was amplified as an internal control. Standard curves were designed using PCR products in known ratios. Gel electrophoresis and image analysis allowed for determination of predicted % Y chromosome-bearing spermatozoa (predicted % Y spermatozoa). Calf sex was reported and % male calves was determined between bull, ejaculate within bull, state, and location within state. Predicted % Y spermatozoa and % male calves showed significant correlation to each other. No significant variance between bull was found in predicted % Y spermatozoa or % male calves, but significant variance was found between ejaculate within bull for both. PCR technology used for determining the % Y spermatozoa in ejaculates was shown to be an adequate method to determine semen sex ratio.

Key Words: Sex ratio, Offspring sex, Semen sexing, PCR, SRY

INTRODUCTION

Sex-sorting of bull spermatozoa has applications for genetic improvement in the dairy industry in which production is the ultimate goal. Therefore, since females are more desirable than males, it would be more profitable for dairy farmers to use sexed semen to produce replacement daughters from genetically superior cows (Hohenboken, 1999).

In the artificial insemination (AI) industry, it is possible to collect, extend, package, and ship billions of sperm cells from one male that will be used to breed thousands of females. This allows for the dispersal of superior genetic material throughout the world. Since the sperm cell determines the sex of the offspring, semen sorted by its ability to produce males or females would be very marketable.

The only consistently successful, nondestructive known approach to sexing spermatozoa is to quantify the nucleic acid DNA in spermatozoa using a DNA-binding dye followed by flow cytometry and cell sorting. The sorting process is based on the fact that the bovine X-chromosome bearing spermatozoan has about 4% more DNA than the Y-chromosome bearing spermatozoan. Despite the success of semen sexing using this method, limitations of the flow cytometry method remain, including being slow (15 million spermatozoa per hour), lowered fertility due to the decrease in the survivability of sperm cells, and the high costs of equipment and skilled personnel and costs of intellectual property (Seidel 1999). Therefore, alternative semen sexing methods have been investigated.

The common acceptable ratio of X- to Y-chromosome bearing sperm cells is 1:1; however, the conventional polymerase chain reaction (PCR) technique was used to

evaluate this ratio in bull semen (Chandler 1998, Chandler 2002). A variation was found between the number of X- and Y-chromosome bearing sperm cells in each ejaculate. If this variation could be further proven, it could call for a few changes in the semen collection protocols that would help to alter the ratio of male to female offspring in favor of the needs of the particular producer.

In the bull semen collection industry, generally 4 to 6 ejaculates are collected weekly. Usually two ejaculates are collected each day, pooled (combined) and processed for freezing. The straws obtained from that combined ejaculate (lot) are packaged, stored, and sold together. A single lot will produce on average about 200 breeding units. Since a variation in the number of X- and Y-chromosome bearing sperm cells was found among lots (Chandler, 1998), all of those straws of semen may contain a skewed ratio of X-or Y-chromosomes bearing sperm cells. If this ratio can be determined using the PCR method, then the straws obtained from that lot might be marketed as sex-evaluated semen. This would give producers the opportunity to increase their chances of getting more offspring of the desirable sex.

The PCR technique has several advantages over the flow cytometry such as the low cost of analysis and the fact that PCR analysis requires only about two straws of semen. This thereby preserves the quality of remaining semen and maximizes its fertility compared to flow cytometry, in which the whole ejaculate is needed for sorting. To make this idea viable, the following experiment was designed to use two types of PCR to analyze ejaculates for their sex ratio. The PCR estimated sex ratio of the semen was compared to the sex ratio of calves resulting from actual breedings .

LITERATURE REVIEW

Sexed Semen Benefits

Since knowledge of the sperm sex ratio would be beneficial for many uses as discussed in the introduction, methods of determining that ratio have been researched. An increased proportion of female offspring would be desirable in dairy cattle production, whereas an increased proportion of male offspring would be desirable in beef cattle production (Madalena, 2004). Madalena calculated the actual value of utilizing sexed semen and found that profitability of sexed semen will depend on economic conditions of individual farms and the cattle industry, but mostly on cost of the sexed semen (2004).

Sex Identification and Problems With Flow Cytometry

Although embryo sexing can be utilized for agricultural applications, Seidel (1999) explained that sex identification in vitro has different uses than sex pre-selection before conception. Sex identification was defined as different than sex pre-selection. The research mentioned that manipulation of the female's ovulation time, environmental conditions, and other factors of the female to determine the sex of the offspring have been proven unreliable. Researchers have observed the separation of X- and Y- bearing spermatozoa to be widely applicable and accurate with flow cytometry. However, Catteano (2004) discovered that conception rates were significantly lower with sexed ram semen using flow cytometry compared to non-sexed semen collected from the same ram. Madalena (2004) found that even a 10% reduction in fertility of sexed semen markedly decreased its profitability. The current experiment will study an alternative semen sexing technique that is less expensive and less detrimental to the semen quality compared to flow cytometry.

PCR as a Semen Sexing Tool

Lobel (1993), using conventional polymerase chain reaction (PCR), discovered a variation between human Y-chromosome bearing spermatozoa (% Y spermatozoa) from 41.9 to 56.7%, with an average of 50.3%. The study reported % Y spermatozoa from 98 ejaculates of 95 men. No analysis was performed to determine if the variation was by ejaculate. Chandler (1998) designed a PCR experiment that evaluated the variation between ejaculates and found a significant difference in the % Y spermatozoa within each ejaculate, which confirmed the Lobel (1993) study. Beckett (1989) utilized a similar yet less accurate procedure to Lobel (1993) and discovered a difference in %Y spermatozoa ranging from 27 to 56.7%.

Chandler (1998) studied ejaculate within bull confirmed by % male calves and male pigs born. Sire was not found to be a significant source of variation, but ejaculate within bull and within boar was discovered to be significantly different. Both PCR results and birth data gave strong evidence that % Y spermatozoa in individual ejaculates within animal varies significantly, which contrasted the common idea that each ejaculate contains 50% Y spermatozoa and 50% X spermatozoa. This also indicated that conventional PCR could be used to determine semen sex ratio.

PCR and Real-Time PCR (RT-PCR) have been utilized to determine sex of embryos and semen. Avery (1992) performed PCR on bovine 7, 8, and 10 day-old blastocysts to compare sex of embryo to growth rate. An average sex ratio of 50% was found using the Y-chromosome-specific gene BRY. Petite (1995) developed a rapid and accurate sexing method for unincubated chick embryos with PCR.

RT-PCR

Paul (2003) utilized conventional and RT-PCR to determine the % X-chromosome bearing spermatozoa (% X spermatozoa) and % Y spermatozoa in bull and boar ejaculates and confirmed some results with piglet sex ratio per litter. Paul (2003) found correlation between piglet data and RT-PCR but no significant correlation was seen between piglet data and conventional PCR results.

Chandler (2002) studied the effect of collection frequency on semen sex ratio using conventional PCR with primers designed to amplify pieces of the Factor IX (Christmas Factor) (FIX) and sex determining region on the Y chromosome (SRY) genes. Primers were used in a duplex PCR reaction on DNA from different ejaculates from the same bull to produce a 151 bp product from the SRY gene and a 122 bp product from the FIX gene. PCR products were electrophoresed through a 4% gel and band intensity/densities were compared to bands of known X and Y DNA ratio.

Although conventional and RT-PCR were used to determine a difference in % X spermatozoa and % Y spermatozoa within an ejaculate (Paul, 2003), more research was needed to study the use of RT-PCR. The ten most common RT-PCR pitfalls were listed on the Ambion website (2004), including excluding the use of master mixes, utilizing poor primer design and cross-contamination. The use of SYBR-Green RT-PCR dye was found to be a less expensive and simple alternative to the use of Taqman probes. Benefits of RT-PCR over conventional PCR were mentioned such as speed, ability to monitor after each replication, and ability to use small amounts of template DNA (Ponchel, 2003). Joerg (2004) compared a piece of the SRY gene to the MSHR autosomal gene using RT-PCR on bovine semen DNA. Conclusions suggested that RT-

PCR is a reliable technique to determine the ratio of % X to %Y bovine spermatozoa. However, the cost of RT-PCR, if used as a tool to analyze the sex ratio of semen, would be significantly higher than the use of conventional PCR. Madelena (2004) found that cost of sexed semen significantly affects the profitability of it. Therefore, RT-PCR would be a much less profitable method of determining sex ratio.

Housekeeping Genes

A housekeeping gene (endogenous control) to be amplified within each PCR reaction was needed for use as a positive control. Wang (2004) used human B-actin as a housekeeping gene in a RT-PCR study determining which genes are responsible for spermatogenesis. Wu (1993) utilized actin as a housekeeping gene. Jafar (1995) used a prolactin PCR product as an internal control while PCR was used to determine blastocyst sex. A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA segment was amplified with PCR and employed as a housekeeping gene (Goodwin, 2000).

Alterations were needed to optimize the conventional PCR techniques to be used in this study. As difficulties were encountered while performing duplex PCR using primers from Paul (2003), many possible problem-causing factors were studied. Henegariu (1994) provided a flow chart format explanation of ways to optimize conventional duplex PCR results. The first step listed was to ensure primers were binding in the correct regions of the DNA. Therefore, alternate X and Y chromosome binding primers were explored.

Primers

Henegariu (2004) discussed essential steps for choosing and designing PCR primers. Parameters listed included designing primers with similar melting temperatures

and a length of 18-24 bp. Mouse preimplantation embryos have been sexed with a 2-step PCR method using SRY and ZFY Y-chromosome specific target sequences (Welch, 1995). Flow-cytometry sorted bull semen was re-analyzed with PCR using primers for the ZFY and ZFX genes. Welch (1995) experienced high numbers of unsuccessful amplification reactions as compared to previously reported human sperm PCR analysis. Jafar (1995) used conventional PCR to yield a 307 bp Y-chromosome specific product from blood and used a prolactin PCR product as an internal control.

Rosel (2003) built a duplex conventional PCR system to simultaneously amplify the SRY gene and the 2FX gene. PCR was altered to allow for a small piece of skin to be used directly as template DNA (Rosel, 2003). Peura (1991) developed a reliable conventional PCR protocol for determining bovine preimplantation embryo sex using 2 bovine Y-chromosome specific primer sets and 1 bovine DNA specific primer set. Conventional PCR was used to amplify the amelogenin (AMEL) locus, which used only one primer pair. The AMEL primer produced only one 280-bp product in the X-chromosome bearing spermatozoa but amplified that and an additional 217 bp product from the Y-chromosome bearing spermatozoa (Madrid-Bury, 2003). Bryja (1995) used a set of primers modified from Pomp (1995) and a set of primers from Sanchez (1996) to amplify a 157-base pair (bp) and a 202-bp region of the SRY gene for sexing various wild mammals. Results found that Pomp's primers (1995) did not work for bovine DNA but this also may have been due to DNA extraction difficulties.

Pomp (1995) was not able to sex cattle using the protocol. Schroder (1990) used Y-chromosome specific primers to sex bovine preimplantation embryos with PCR.

Since alternate products decrease primer efficiency and could produce false results, it was important that primers chosen for this experiment amplified only single-copy genes. Bryja (1995) confirmed that the SRY gene is a single-copy gene found in most mammals. The National Center for Biotechnology Information (NCBI) website (2004) confirmed that the Factor IX (Christmas Factor) is a single-copy gene found on the X-chromosome.

PCR Thermocycler Temperatures

A second step discussed by Henegariu (1994) was the alteration of denaturing, melting, and annealing temperatures and duration times. The researchers studied many methods to determine optimum cycling conditions for duplex PCR. Rychlik (1990) described the importance of annealing temperature (T_a) optimization when amplifying either long PCR products or using genomic DNA as a conventional PCR template. A mathematical calculation for the optimum T_a (T_{aOpt}) was established, creating T_a as a function of the less stable primer-template pair's melting temperature (T_m) and of the PCR product. The project demonstrated that if T_a is below T_{aOpt} , non-specific bands were replicated.

DNA Extraction

In this experiment, DNA extraction was a source of possible problems as well. Arivanidakshan (1998) developed and published a method for extracting bovine semen genomic DNA using a method including the use of a lysis buffer, proteinase K, phenol chloroform, and other reagents. Paul (2003) successfully used the Arivanidakshan lysis solution to extract DNA from bovine semen.

MATERIALS AND METHODS

Calving Data

The purpose of this experiment was to confirm previous findings of a variation in the ratio of X- and Y-chromosome bearing sperm cells between bovine ejaculates.

A total of 39 ejaculates from eight Holstein bulls were collected at two separate semen collection facilities (GENEX Cooperative, Inc. and Select Sires, Inc.). Six ejaculates from each of 4 bulls and 3 ejaculates from each of 4 other bulls were collected. Approximately 30 million spermatozoa were stored in each straw, frozen with current commercial freezing techniques, and stored in liquid nitrogen. Ejaculates from GENEX Cooperative, Inc. were diluted in a milk-based extender, while ejaculates from Select Sires, Inc. were diluted in an egg-based extender. Straws of semen from the ejaculates were sent to dairies in Mississippi, New York, and Louisiana for breeding purposes. Cows were bred with artificial insemination techniques and as calves (n=540) were born, the sex of the calf was reported back to researchers at Louisiana State University (LSU) for statistical analysis. Ten straws from each ejaculate were sent to the LSU Dairy Improvement Center for Polymerase Chain Reaction (PCR) and quality control analysis.

Semen Quality Control

Two straws of frozen semen from each ejaculate were thawed and analyzed. Percent progressive motility was evaluated from a live smear using phase contrast microscopy equipped with a warm stage at 40X magnification at 0 hours and again after a 3-hour incubation at 37°C (Saacke, 1972). Primary and secondary abnormalities were determined by direct count of 100 cells from random fields of an immobilized smear using differential interference contrast (DIC) microscopy at 100X magnification (Mitchell, 1978). Acrosomal integrity, denoted by the presence of the apical ridge after 3

hours of incubation at 37°C, was measured using DIC microscopy at 100X magnification counting 100 cells in an immobilized smear (Saacke, 1972). All immobilized smears were fixed with .02% glutaraldehyde (Johnson, 1976).

DNA Extraction and Quantification

DNA was extracted using a protocol from Arivanidakshan (1998). Fresh lysis solution (Arivanidakshan, 1998) was prepared before beginning DNA extraction. The lysis solution was made in two steps. First, 0.121 g Tris (pH = 8.0, 10 mM), .0293 g EDTA (pH = 8.0, 10 mM), and 80 mL distilled water were brought to pH = 8.0 using NaOH. Second, 0.584 g NaCl, 2 mL 2-mercaptoethanol and 0.5 g SDS were added, volume was brought to 100 mL with distilled water, and the solution was stored at 2-8°C.

Two straws of each ejaculate were thawed at room temperature. Sperm cells were emptied into labeled 1.5 mL centrifuge tubes. Tubes were then centrifuged at 15,000 g for 3 minutes and the supernatant was discarded.

Sperm cells stored in milk underwent an additional washing step in which they were resuspended in 500 µL of sodium citrate, spun for 3 minutes at 15,000 g and supernatant was discarded. Each pellet was resuspended with 500 µL Arivanidakshan lysis solution, warmed to 50° C and vortexed to ensure contact. Samples were incubated at 50° C for 30 minutes and then 5 µL of proteinase K was added and mixed gently. The tubes were incubated overnight in a water bath warmed to 50° C. After 14-18 hours, 500 µL of phenol chloroform was added to each sample and shaken often while sitting at room temperature for 15 minutes. Samples were centrifuged for 3 minutes at 15,000 g and the bottom layer was removed by Pasteur pipette. The phenol chloroform step was repeated once again. Then 500 µL of chloroform was added to each sample and shaken

often at room temperature for 10 minutes. The samples were centrifuged for 10 minutes and the bottom layer was discarded. Ice cold absolute ethanol (1000 μ L) was added to each sample and swirled in a circular motion. White or viscous threads were observed at this point. Tubes were centrifuged for 1 minute to pellet the DNA at the bottom of the tube. Excess ethanol was poured off the top and the pellets were desiccated in a vacuum desiccator for approximately 1.5 hours. Many times the pellet was clear or invisible. Samples were then resuspended with 300 μ L molecular grade water and incubated at 37° C for approximately 2 hours. The DNA was stored in a freezer at -20° C until PCR analysis.

The DNA was quantified by measuring absorbance at 260 nm using a Spectronic® Genesis™ 5 Spectrophotometer (Spectronic Instruments, Rochester, NY). The measurement was used to quantify the amount of DNA in each tube, and then calculations were made to ensure that 150 μ L template DNA was used for each PCR reaction. To do this, each 260 nm measurement was divided by the X coefficient (.022), which was derived from a standard curve using calf thymus DNA (Sigma, St. Louis, MO), and then that product was divided into 150 to determine the number of μ L of DNA to place into each tube. For example:

260 nm absorbance = 1.54
 $1.54/.022 = 70$
 $150 \text{ ng}/70 = 2.14 \mu\text{L DNA}$ into the reaction

Conventional PCR

PCR tubes (.5 mL) containing the PCR beads (puReTaq Ready-To-Go PCR Beads, Amersham Biosciences, Piscataway, NJ) were used to eliminate problems of PCR component concentrations other than DNA and primers. PCR reactions were prepared in

sets of 20 or 30 according to the size of gel being used to ensure clarity of bands and to eliminate the risk of PCR product denaturation. This was also to allow for all controls and as many ejaculates as possible to be combined onto one picture to decrease gel variability during analysis. The DNA of each ejaculate was amplified using PCR in triplicate. Figure 1 following is an example of how PCR was set up.

After each PCR run, the X DNA tubes were combined and the Y DNA tubes were combined to create a pooled version of that PCR product to use for the DNA of known X and Y ratios.

Target Sequence and Primer Design

An internal housekeeping gene was needed as a positive control. Segment 367-660 of the *Bos taurus* glyceraldehyde-phosphate-dehydrogenase (GAPDH mRNA, accession # U85042 (NCBI, 2005), located on chromosome 5, Locus 12p3 (ARKdb, 2000)) was chosen as the segment to amplify because of its homologous qualities with human GAPDH (accession # M17851) successfully used by Goodwin (2000). Vector NTI™ 5 software (InVitrogen™) was used to design primers. Primers were chosen according to product length, GC content and Tm (Henegariu, 2004).

Primers were synthesized at the LSU Gene Probes and Expressions Systems Laboratory (Baton Rouge, LA). GAPDH was tested to ensure its quality and accuracy. The GAPDH primers were predicted by the designing software to produce a product of 269 bp.

GAPDH primer sequences were as follows:

GAPDH S: 5' – CAC CCT CAA GAT TGT CAG CA - 3'
Tm: 50.9° C
GC Content: 50%

GAPDH A: 5' CCA TGC CAG TGA GCT TCC - 3'
Tm: 50.9
GC Content: 61.1

Primers designed by Paul (2003) were used to amplify sections of the X chromosome Factor IX gene (BOVFIX primers) and a section of the SRY gene on the Y chromosome (SRYB50 primers). The primers were originally purchased from the LSU Gene Probes and Expressions Systems Laboratory (Baton Rouge, LA). However, due to lack of reproducibility, this experiment's BOVFIX and SRYB50 primers were purchased from Integrated DNA Technologies, Inc. (IDT). Primer sequences were as follows:

BOVFIX S: 5' - TCG AAC ATG CAG CAT TAA GA – 3'
Tm (50mM NaCl): 53.0° C
GC Content: 40.0 %

BOVFIX A: 5' - GTT CGC AGG ACT TTT GGT CT - 3'
Tm (50mM NaCl): 55.4° C
GC Content: 50%

SRYB50 S: 5' – CTT CAT TGT GTG GTC TCG T – 3'
Tm (50mM NaCl): 52.4° C
GC Content: 47.3%

SRYB50 A: 5' – TAG TCT CTG TGC CTC CTC A – 3'
Tm (50mM NaCl): 54.5° C
GC Content: 52.6

The resulting PCR products from the IDT primer sets used for this experiment were sequenced and a database search was used to determine the size and location of the actual PCR product being amplified with the BOVFIX and SRYB50 primers.

Setting Up Conventional PCR

After much trial and error optimization, a primer concentration of 12.5 μ M and a template DNA concentration of 150 ng DNA per reaction was used.

TUBES		DNA	μL BOVFIX A	μL BOVFIX S	μL SRYB50 A	μL SRYB50 S	μL GAPDH A	μL GAPDH S	μL H2O
X ONLY	POOL	5.26	2	2					15.74
X ONLY	POOL	5.26	2	2					15.74
X ONLY	POOL	5.26	2	2					15.74
Y ONLY	POOL	5.26			2	2			15.74
Y ONLY	POOL	5.26			2	2			15.74
Y ONLY	POOL	5.26			2	2			15.74
GAPDH	POOL	5.26					2	2	15.74
DNA only	POOL	5.26							19.74
Primers only			2	2	2	2			17
POOLED	POOL	5.26	2	2	2	2			11.74
Bull 1	A1	1.43	2	2	2	2			15.57
Bull 1	A2	1.43	2	2	2	2			15.57
Bull 1	A3	1.43	2	2	2	2			15.57
Bull 1	B1	1.39	2	2	2	2			15.61
Bull 1	B2	1.39	2	2	2	2			15.61
Bull 1	B3	1.39	2	2	2	2			15.61
Bull 1	C1	1.45	2	2	2	2			15.55
Bull 1	C2	1.45	2	2	2	2			15.55
Bull 1	C3	1.45	2	2	2	2			15.55
Bull 1	D1	1.62	2	2	2	2			15.38
Bull 1	D2	1.62	2	2	2	2			15.38
Bull 1	D3	1.62	2	2	2	2			15.38
Bull 1	E1	2.94	2	2	2	2			14.06
Bull 1	E2	2.94	2	2	2	2			14.06
Bull 1	E3	2.94	2	2	2	2			14.06
Bull 1	F1	2.95	2	2	2	2			14.05
Bull 1	F2	2.95	2	2	2	2			14.05
Bull 1	F3	2.95	2	2	2	2			14.05

Figure 1. PCR setup.

Two μL of each sense and each antisense primer was used in each tube, totaling 4 μL primers. After DNA was added, the total solution was brought up to 25 μL. Samples were covered with 50 μL mineral oil to eliminate evaporation.

Utilizing suggestions from Henegariu (1997), the duplex PCR reaction used by Paul (2003) was optimized by adjusting thermocycler temperatures and times. The following thermocycler settings were used:

1 cycle - initial DNA denaturation

95 C for 7 minutes (2 minutes for heating up, held at 95 C for 5 minutes)

29 cycles – denaturation, melting, and annealing

1 minute at 95 C

45 sec at 51 C
1 min at 68 C

1 cycle – final annealing
5 min at 68 C

Hold
Hold at 9 C

Preliminary Trials to Create DNA of Known X and Y Ratios

DNA with known X and Y product ratios was needed to use as controls for unknown comparisons. Large quantities of the products were needed in order to use the same controls for all gels.

PCR product produced from the BOVFIX primer set amplifying a section of the X-chromosome was referred to as X DNA because the primers amplified a section of the X-chromosome. PCR product produced from the SRYB50 primers were referred to as Y DNA because the primers amplified a section of the Y-chromosome. X DNA and Y DNA were needed to produce standards of 20%X /80%Y, 40%X /60%Y, 60%X /40%Y, and 80%X /20%Y.

An attempt to create mass amounts of X and Y products was done by performing PCR with the specific SRYB50 and BOVFIX primers, separating the PCR products by gel electrophoresis, extracting the DNA out of the gel bands, and then performing PCR again on that gel-extracted DNA. After many trials, this method was determined unsuccessful.

For a second approach, due to recommendations from personal communication with Cooper (2005), the product of interest was inserted into a TOPO cloning vector. Duplex PCR was performed with pooled DNA samples of all ejaculates. The X and Y bands were separated on an agarose gel and the gel bands were cut out. Gel extraction

produced pure X and Y products. Products were inserted into a cloning vector using the TOPO TA® Cloning Kit (InVitrogen™) and the vector was placed into a strain of *E.coli*. The bacteria was grown and the vector DNA was extracted from the *E.coli*. These also could not be used as accurate control DNA.

The third and successful approach to obtaining mass amounts of DNA for use in creating DNA of known X and Y ratios was to simply combine PCR products in ratio amounts. After DNA was extracted from every ejaculate, 20 µL from each ejaculate's DNA was added to one common microfuge tube to create a true pooled sample. This pooled DNA was used to create the X and Y DNA to be used. For each set of PCR reactions, 3-4 reactions containing only the BOVFIX (X) primers and 3-4 reactions containing only the SRYB50 (Y) primers were prepared and run alongside the samples. After PCR, the products were combined into known ratio amounts to produce the lanes with a known DNA ratio.

The following is an example of how the tubes of DNA with known ratios were prepared:

X DNA
2 µL PCR product

Y DNA
8 µL PCR product

Total = 20% X DNA, 80% Y DNA

Gel Electrophoresis

A 3% agarose I (Agarose I, Amresco®, Solon, Ohio) gel was made. Three grams of agarose were added to 100 mL 1X TAE. Thirty-well combs were used for most samples. Other samples were loaded into 20-well comb gels, depending on the

availability of laboratory equipment each day. Ten μL of each PCR product were loaded into each well regardless of well size. Controls constituting ratios of 20X/80Y, 40X/60Y, 60X/40Y, and 80X/20Y were included in each gel on the outer edges, and samples were arranged in triplicate within the gel. A 25 bp marker was included to show the location of each band in the samples, and a GAPDH PCR product was included in every gel as an internal control.

Gels were electrophoresed at 120 volts for approximately 3 hours. Gels were then stained in an ethidium bromide solution (0.5 $\mu\text{g}/\text{mL}$) and pictures were taken with the ChemiDoc™ XRS System (BIO-RAD Laboratories, Inc.) using approximately 1.5 second shutter exposure.

Picture Analysis

Pictures were analyzed using Quantity One® Software 4.1 (BIO-RAD Laboratories, Inc.). Lanes were framed and bands were detected using Gaussian band detection. Band detection sensitivity was adjusted for each gel and unneeded bands were deleted from the analysis. All lane reports were produced and exported into spreadsheets (Figure 2) for statistical analysis. Pictures were then labeled and image reports (Figure 3) were utilized to organize the data spreadsheets. Trace quantity (in units of intensity x mm) was collected as the response indicative of the amount of X- and Y-chromosome DNA represented by each band. Trace quantity was defined by the program as being the quantity of a band measured by the area under its intensity profile curve.

Statistical Analysis

Since for unknown reasons the 122 bp X PCR product did not amplify correctly

and therefore was not significantly visible on the gel but the 151 bp Y product did amplify correctly, the Y product was used for analysis. An inverse regression of the Y product band trace quantity across lanes in the gel was used to create a standard curve (Ryan, 1997). Each unknown %Y in each lane was determined by calculating the logratio of trace quantities of the bands in a particular lane. Percent Y was then calculated by taking the logratio and using the formula: $\%Y = a + b(\text{logratio})$, when $a =$ intercept, $b =$ slope of the standard inverse regression curve, and $x =$ logratio of the band trace quantities.

All data (semen quality, %male calves and %Y DNA) were analyzed with PROC GLM of SAS[®] with the appropriate statistical mixed model (SAS[®], 2001). The model effects were tested with the appropriate error terms by using the random statement with the test option and required by expected means squares (Steel, 1980).

RESULTS

Percent Male Calves

Personnel from each state's Agricultural Experiment Station (Louisiana and Mississippi) chose the bulls for use in their state. Genetic improvement of each dairy's calves was used as a deciding factor when technicians decided which bull to use for breeding. Semen from 4 bulls was purchased for use in Louisiana and semen from 5 bulls were purchased for use at the Mississippi location, but the number of bulls used totaled 8 because there was one common bull between the two states. Three ejaculates from each of 4 bulls were used for the experiment, whereas six ejaculates were used from each of the other 4 bulls. Between 24 and 42 units of semen from each ejaculate were distributed to the state that purchased them. Then the units of semen were dispersed to different locations within each state.

Semen used in Louisiana was collected solely for this experiment. Therefore, the collection facility that provided semen for Louisiana had some remaining units after distributing between 24 and 48 units per ejaculate. Those remaining straws were sent only to private dairies in New York with histories of routinely and faithfully reporting the sex of calves.

Since one bull produced only 11 calves across all of his ejaculates, he was culled from the study, thus the analyses were performed with 7 bulls instead of 8 (Table 1). Ejaculates that did not produce at least 8 calves were excluded from the data, so only 23 ejaculates were used for statistical analysis. Some calf sex data was lost because one herd in Mississippi was dispersed. An average of 44 calves were born at each location and there was an average of 20 calves produced per ejaculate.

In Table 3, bull (P=0.4980), parity (whether the dam was a first-calf heifer or a cow) (P=0.5315) and state (P=0.8654) showed no significant effect on variation in % male calves. The variation in % male calves attributed to ejaculate within bull was significant (P=0.0967). Location within state was also found to influence the variation in % male calves (P=0.0058). This is evidenced by the % male calves values for the Mississippi location #3 and for the New York location #6 (Table 4).

Table 1. Analysis of variance of percent male calves for the LA, NY and MS sex ratio breeding study.

Source	DF	Sums of Squares	Mean Square	F Value	Pr > F
Corrected total	484	121.1876289			
Bull	6	1.787233	0.297872	0.92	0.4980
Residual†	26.403	8.571838	0.324648		
†Residual: 0.8799*MS(Ejaculate(bull)) + 0.1201*MS(Residual)					
Ejaculate(bull)	22	7.407574	0.336708	1.43	0.0967
**State	2	0.068340	0.034170	0.14	0.8654
Location(State)	8	5.182182	0.647773	2.74	0.0058
Parity	1	0.092661	0.092661	0.39	0.5315
Residual: MS(residual)	445	105.143690	0.236278		
** This test assumes one or more other fixed effects are zero.					
R ² (%)		13.23	Mean % Male calves		51.13

Table 2. Least squares estimates of percent of male calves, standard error and number of calves by location within state.

Location	State	Male calves (%)	Standard Error	Number of Calves
1	LA	49.8%	8.4%	97
1	MS	42.0%	10.5%	42
2	MS	52.5%	14.2%	20
3	MS	27.8%	17.8%	11
4	MS	45.4%	7.6%	126
1	NY	41.8%	9.9%	51
2	NY	51.0%	11.5%	30
3	NY	55.0%	9.9%	48
4	NY	28.7%	14.0%	19
5	NY	26.4%	15.6%	14
6	NY	87.6%	12.1%	27

Figure 4 illustrates the range for the distribution of % male calves per ejaculate within and across bulls. Two ejaculates yielded only 25% male calves and two others yielded 65% males.

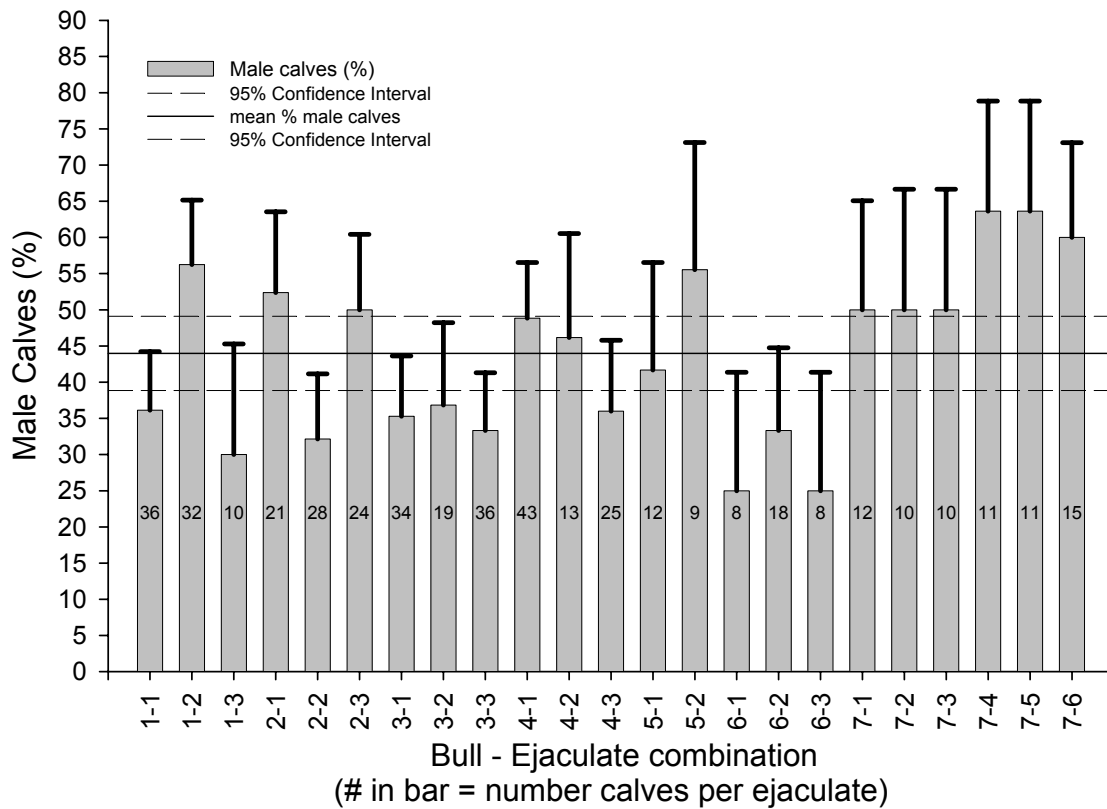


Figure 4. Percent Male Calves per Ejaculate

PCR Product Sequences

When designed, BOVFIX primers were expected to result in a 122 bp product, whereas SRYB50 had an expected result of a 151 bp product (Paul, 2003). However, Paul's PCR products were never sequenced. After sequencing the PCR products amplified by the BOVFIX and SRYB50 primers, BOVFIX primers were found to amplify a 125 bp section of the *Bos taurus* factor IX (Christmas factor) precursor,

accession # J00007, located on the q arm between the 32 and 33 position of the X chromosome (NCBI, 2005). The X PCR product sequence was as follows:

```
ATTGCGACAGCAACATTGTCTAGTCATAGACGAGGCACTACAGATCACACAA  
TGGTCGCGTGTCTAGATATCCACTGCTACTGACGGATACCGACTTGCAGAAG  
ACCAAAAGTCCTGCAAACAAT
```

SRYB50 PCR product sequencing resulted in the amplification of a 130 bp section of the *Bos taurus* sex determining region Y protein (Sry) gene, accession # AF148462 (NCBI, 2005), located on the Y chromosome. The Y product sequence was as follows:

```
GAAAGGTGGCTCTAGAGAATCCCAAATGAATTA ACTCAGACATCAAGGCAA  
GCAGCTGGGATATGAGTGGAAAAGGCTTACAGATGCTAGAAAAGCGCCCAT  
CTTGAGGAGGCACAGAGACTAATGA
```

Obtaining DNA of Known X and Y Ratios

An attempt to create mass amounts of X and Y products was done by were originally thought to be obtainable by performing PCR with the specific SRYB50 and BOVFIX primers, separating the PCR products by gel electrophoresis, extracting the DNA bands out of the gel, and then performing PCR again on that gel-extracted DNA. However, after many trials to do so, this method provided little to no successful results. It was later discovered that such an experiment has rarely produced successful results in other LSU laboratories (Cooper, 2005).

After the product of interest was inserted into a TOPO cloning vector and gel analysis of the vector DNA was performed, it was discovered that the vector actually inserted concatomers of the X and Y products that varied in base pair (bp) length into itself. These could not be used for accurate control DNA.

Although both X and Y PCR product DNA were inserted into the ratio controls, for unknown reasons the 122 bp X PCR product did not amplify correctly and therefore was not significantly visible on the gel. However, the 151 bp Y product did amplify correctly, and that product was used for statistical analysis.

Predicted % Y Spermatozoa

The analysis of variance in predicted % Y spermatozoa is presented in Table 5. One ejaculate from each of two bulls was culled from the predicted %Y spermatozoa data because they were determined to be statistical outliers. The analysis showed no significant effect of bull (P=0.1199) or replicate (P=0.861) but did show a significant effect of ejaculate within bull (P=0.0042) (Figure 5).

Table 3. Analysis of Variance for Predicted %Y spermatozoa

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Corrected Total	102	30492.47			
Bull	7	5474.75	782.11	1.83	0.1199
Residual†	28.62	12235	457.52		
†Residual: 1.0148*MS(ejaculate(bull)) - 0.0148*MS(Residual)					
Ejaculate(bull)	29	12298	424.08	2.22	0.0042
Replicate	2	977	28.69	0.15	0.861
Residual:	64	12242	191.29		
MS(Residual)	R ²	60.00			

Table 4 shows the correlation of the following semen quality measurements: percent progressive post-thaw motility (PPMO), percent progressive 3-hour motility (PPM3), 3-hour percent intact acrosomes (PIA3), percent primary abnormalities (PPAbn), and percent secondary abnormalities (PSAbn). None of the semen quality parameters showed much impact on % male calves or predicted % Y spermatozoa except

PIA3, which had significant ($P=0.0048$) correlation with % male calves and significant ($P=0.0735$) correlation with predicted % Y spermatozoa. The correlations of semen quality to % male calves and predicted % Y spermatozoa were based upon a single semen quality observation across all ejaculate/bull combinations.

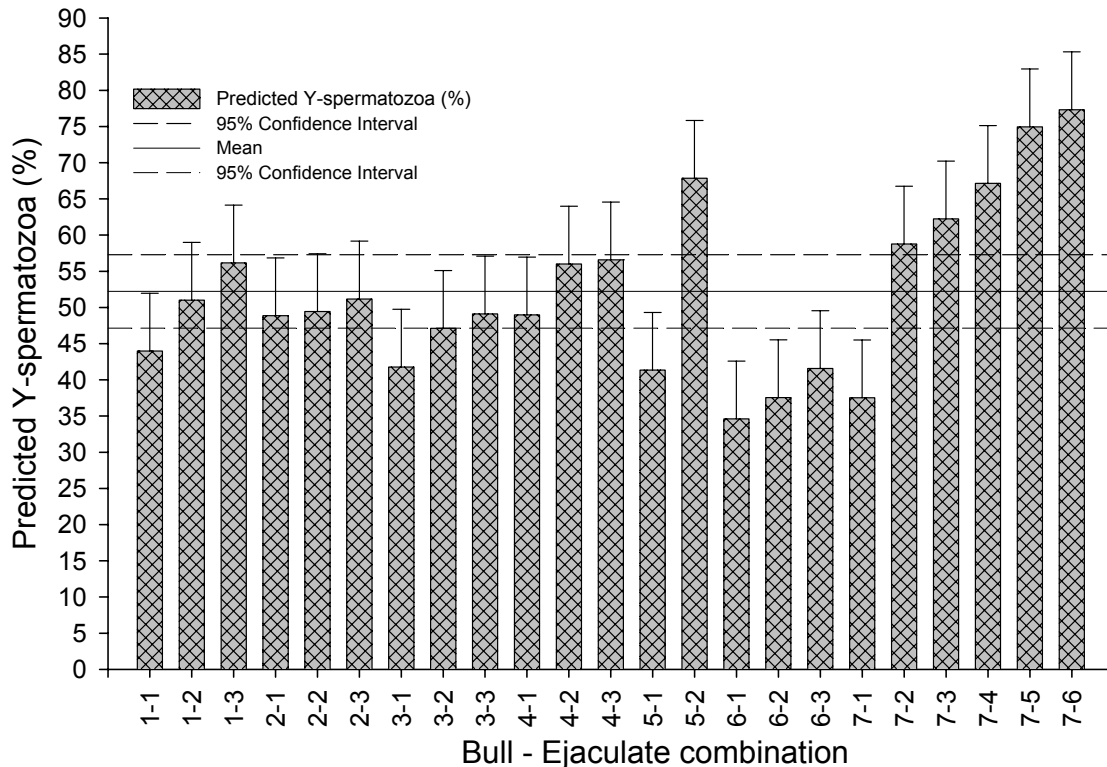


Figure 5. Predicted % Y Spermatozoa per Ejaculate

No correlation was found between 3-hour motility and 3-hour acrosome integrity. Primary and secondary abnormalities showed a negative correlation with each other ($P=0.0050$). These are not measurements of viability but of morphology. Pearson product moment correlation analysis was performed across all ejaculates on the least square means of predicted %Y spermatozoa and % male calves. The % male calves means were corrected for location within state bull and parity. The predicted %Y

spermatozoa means were corrected for bull and replicate. When comparing the predicted %Y spermatozoa to % male calves, a significant correlation was found (P=.0002). This relationship is illustrated in Table 6 and both Figures 6 and 7.

Table 4. Pearson correlation coefficients (r) between sex ratio characteristics [% male calves (P_M_calves_lsm), predicted % Y spermatozoa (PredPtY_LSM)], and semen quality characteristics [percent progressive motility post thaw and before (PPM0) and after (PPM3) a 3hr – 37°C incubation; percent intact acrosomes after a 3hr – 37°C incubation (PIA3); percent primary abnormalities (PPAbn) and percent secondary abnormalities (PSAbn)].

Correlation coefficient (r) Pr > r under H ₀ : Rho=0						
Number of observations	PredPtY_LSM	PPM0	PPM3	PIA3	PPAbn	PSAbn
Pmale_calves_lsm	0.70806 0.0001 24	0.01434 0.9470 24	-0.07707 0.7204 24	0.57829 0.0048 22	-0.11780 0.5924 23	-0.08855 0.6879 23
PPtY_LSM		0.00785 0.9709 24	-0.25390 0.2312 24	0.38905 0.0735 22	-0.11039 0.6161 23	-0.07655 0.7285 23
PPM0			-0.28364 0.1792 24	-0.03018 0.8939 22	-0.05640 0.7982 23	-0.11843 0.5905 23
PPM3				0.04675 0.8363 22	0.33567 0.1174 23	0.18780 0.3908 23
PIA3					-0.25486 0.2524 22	-0.18466 0.4107 22
PPAbn						-0.56454 0.0050 23

Table 5. Regression Analysis of Variance for the Relationship of Predicted % Y spermatozoa (PredPtY) to % Male Calves (P_M_calves).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Corrected Total	22	3097.87			
Model	1	1548.79	1548.80	21.00	0.0002
Residual	21	1549.07	73.76		
R ² (%)		50.00	P_M_calves Mean (%)		43.96
Parameter	Estimate	Standard Error	t Value	Pr > t	
Intercept	6.6	8.3	0.79	0.4381	
PredPtY	0.71	0.15	4.58	0.0002	

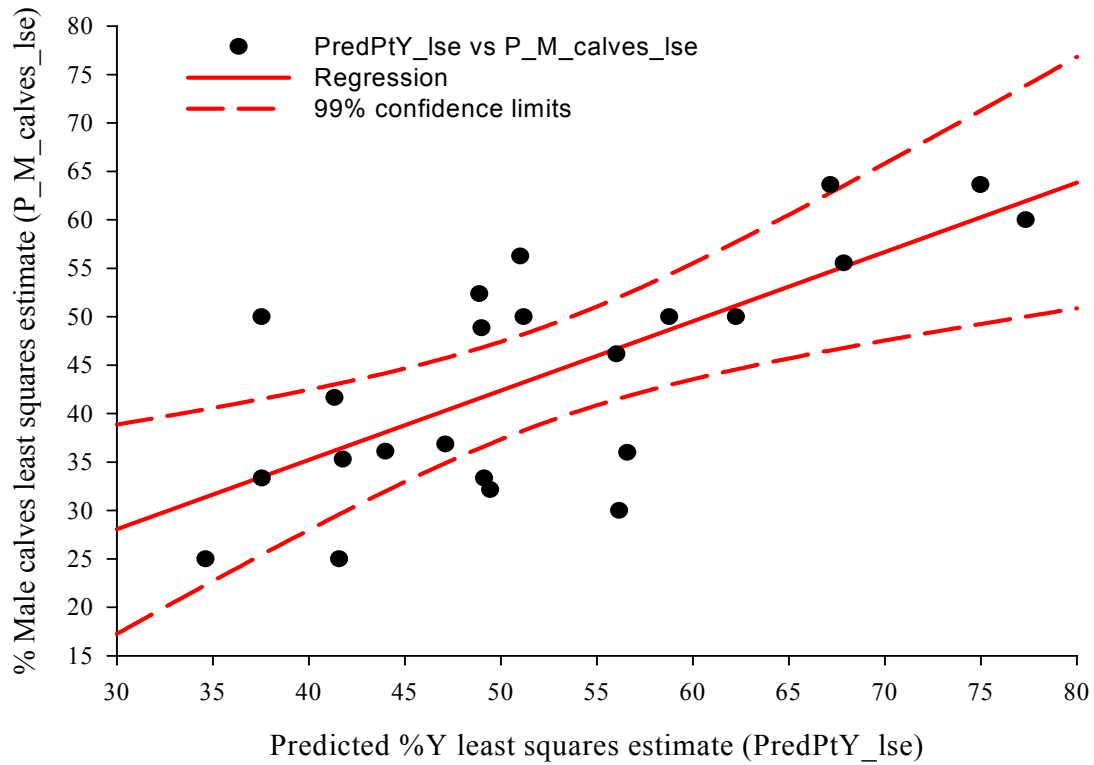


Figure 6. Regression analysis of % male calves (P_M_calves_lse) in comparison with predicted % Y spermatozoa (PredPtY_lse).

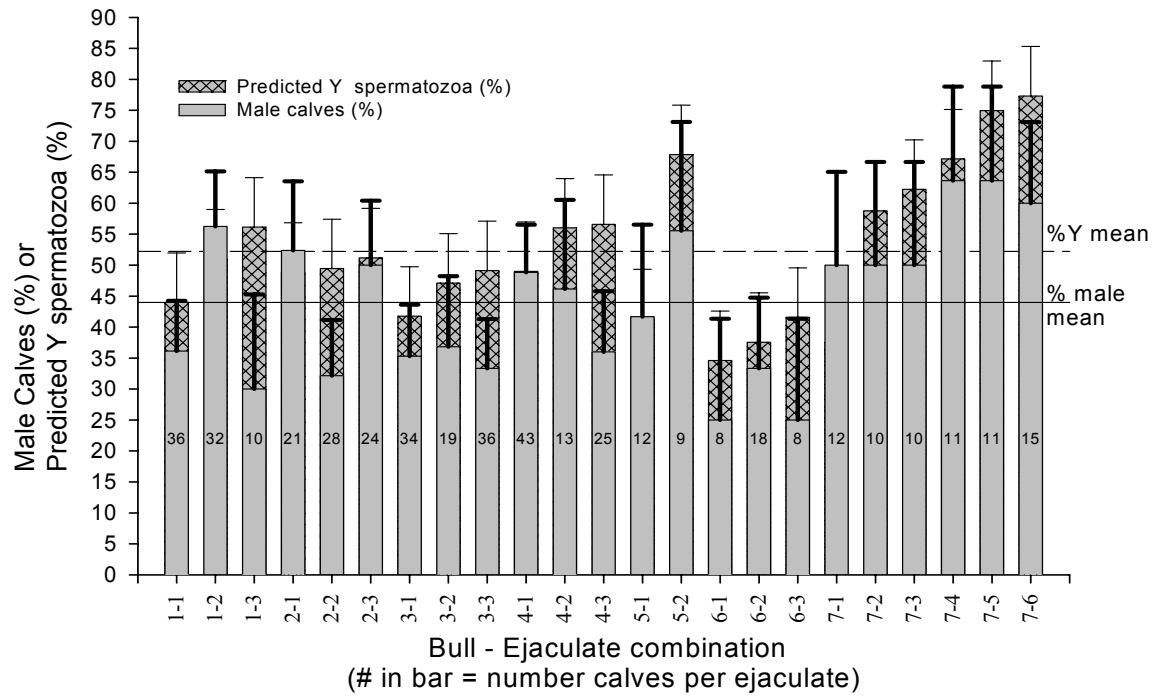


Figure 7. Correlation of % Male Calves and Predicted % Y Spermatozoa

DISCUSSION AND CONCLUSION

Percent Male Calves

The analysis of variance determined that there was no significant effect of bull, parity, or state on the % male calves. However, as reported by Chandler (1998), a significant difference in % male calves between ejaculates per bull was found. Results support the previous observations that ejaculates vary in % male calves (Chandler, 1998), but when multiple ejaculates are evaluated within bulls, the % male calves average across bulls do not differ from the expected 50% sex ratio. The lack of significant bull influence on % male calves disputes the idea that one bull commonly produces a higher percentage of either male or female calves.

Predicted % Y Spermatozoa

Similar results were found with the predicted % Y spermatozoa determined with conventional PCR. Analysis of variance showed no significant bull effect. Replicated PCR runs of the same ejaculate produced no significant variation, which means there was little error in the PCR, gel electrophoresis, and picture analysis procedures. A significant variance was seen in ejaculate within bull, which again supports research conducted by Paul (2003) and Chandler (1998). Results support the observation that ejaculates vary in % Y spermatozoa (Paul, 2003) but when multiple ejaculates are evaluated within bulls, the % Y spermatozoa average across bulls do not differ from the expected 50% sex ratio.

Semen Quality

A significant correlation was observed between percent intact acrosomes after a 3-hour 37° C incubation (PIA3) compared to % male calves and predicted % Y

spermatozoa. Semen quality analysis was based on one evaluation per ejaculate/bull combination, which means that the data were not least squares means estimates. The correlation of semen quality within % male calves and predicted % Y spermatozoa could possibly reflect an influence on fertility. While Saacke (1972) reported significant correlation between 2-hour acrosome integrity and 2-hour motility, our data set does not show any significant correlation between the two. This could be due to our lab's procedure of taking 3-hour acrosome integrity and 3-hour motility measurements, compared to the 2-hour acrosome integrity and motility observed by Saacke (1972). The semen quality data are auxiliary to the general idea of the experiment, but this correlation may be an issue for further research. It must be noted, however, that this experiment's semen quality data did not indicate any significant influence of motility on % male calves or predicted % Y spermatozoa, which refutes many conjectures that Y-spermatozoa swim faster than X spermatozoa.

Percent Male Calves and Predicted % Y Spermatozoa

When the % male calves was compared to the predicted % Y spermatozoa, a correlation was found. Therefore, conventional PCR using primers from this experiment would be a practical method for determining the % Y spermatozoa in an ejaculate. This is in disagreement with the lack of correlation between conventional PCR and farrowing data reported by Paul (2003).

The correlation graph (Figure 6) shows the regression analysis of % male calves on % Y spermatozoa. This regression shows the 99% confidence limits of the prediction of % male calves from % Y spermatozoa found in different ejaculates. This indicates that when an ejaculate is evaluated by PCR techniques and found to have a high level of % Y

spermatozoa that there is a 99% level of probability that use of this ejaculate would result in a shift in the resulting calving sex ratio (% male calves). However, the accuracy of this prediction is limited based upon three things: the strength (correlation) of the relationship between % Y spermatozoa and % male calves; the extent to which events governing the occurrence of the relationship have changed; and the extent to which the data are representative of the relevant population. The correlation between predicted % Y spermatozoa and % male calves was 0.71 which gave an accuracy of 50% (R-square of the regression model, Table 5). The accuracy of the prediction is illustrated in Figure 6 in that 12 of the 23 observations (52%) lay within the 99% confident limits.

Even with the omission of one entire bull's data (due to a low number of calves born) and two ejaculates, the variance found in ejaculate within bull was well supported with 445 calf observations and 102 predicted % Y spermatozoa observations with replicates.

Difficulties and Suggestions for Further Research

Difficulties in this experiment arose in primer designing, PCR optimization, and obtaining the known ratios of X and Y DNA. A 130 bp product for the Y product and a 125 bp product for the X product were shown by sequencing the PCR products of the BOVFIX and SRYB50 primers. This differs from the expected products originally designed by Paul (2003) and from the approximate bp size determined by position on the agarose gel as compared to a marker. However, products adequately separated on the 3% agarose gel and were easy to analyze. Analysis of % Y spermatozoa within ejaculates could probably be performed more quickly and efficiently with primers that amplify products with more bp, so that a 2% or 1% agarose gel could be used and time of gel

electrophoresis could be decreased. The Factor IX (Christmas factor) and the SRY genes were adequate genes to amplify for % Y spermatozoa content.

During this experiment, it was learned that conventional PCR conditions need to be optimized for each different template DNA. Genomic DNA must be denatured for a longer period of time. The flow chart reported by Henegariu (1994) was found to be an accurate method to target and solve many problem-causing agents within the conventional PCR system.

For future research, the BOVFIX primer set should be re-designed. Although the BOVFIX primers amplified the unknown products effectively, they did provide problems throughout the project until the end when they did not amplify the desired DNA section within the pooled DNA that was to be used as the DNA of known ratio content. Possible causes for BOVFIX primer efficiency problems include GC content, T_m , or multiple binding sites.

Applications

Any notion of difference in % male calves by state was disputed by the distribution of semen across three states. The correlation between location within state could have been skewed because one dairy only provided information for 11 calves.

The experiment was designed to be accurate as well as practical. Semen collection and freezing was performed in commercial semen collection facilities by each business's protocol. PCR techniques were relatively inexpensive and reproducible with minimal laboratory equipment.

Calculations determined that the approximate cost to analyze each ejaculate equals \$7.39 per gel lane. This includes cost of agarose, Ready-to-go PCR tubes, and

chemicals involved in the DNA extraction process. To analyze one ejaculate in triplicate with the four lanes of known % X and Y DNA ratio, cost would approximately equal \$51.73. Cost could be reduced by analyzing more than one ejaculate at a time (making best use of the lanes of known % X and Y DNA ratio) and by analyzing in duplicate instead of triplicate.

Conclusions

In conclusion, this experiment confirmed previous research with well-supported findings, sound statistical analysis, and adequate observations. It was confirmed that conventional PCR can be used to analyze the sex ratio of a given ejaculate. Approximately 60 million spermatozoa (2 straws) were needed to produce the sex ratio data with PCR. PCR could be utilized in the semen collection and distribution industry, in which many straws of semen are produced from one ejaculate. Two of those multiple straws could be used for analysis, while the others from that ejaculate could be marketed with known % Y spermatozoa content with no alteration of semen quality.

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APPENDIX

Sequences

J00007

Bos taurus factor IX (Christmas factor) precursor

Bos Taurus

Factor IX: 181 tactgacggataccgacttgcagaagacccaaaagtcctgcgaaac 224

|||||

X PCR Product: 79 tactgacggataccgacttgcagaagacccaaaagtcctgcaaac 122

AF148462

Bos taurus sex determining region Y protein (Sry) gene

SRY: 1249 gaaaggtggctctagagtatccccaaatgaaa-aactcagacatca--gcaagcagctgg 1305

|||||

Y PCR Product: 1 gaaaggtggctctagagaatccccaaatgaattaactcagacatcaaggcaagcagctgg 60

SRY: 1306 gatatgagtggaaaaggcttacagatgct-gaaaagcgcccattctttgaggaggcacag 1364

|||||

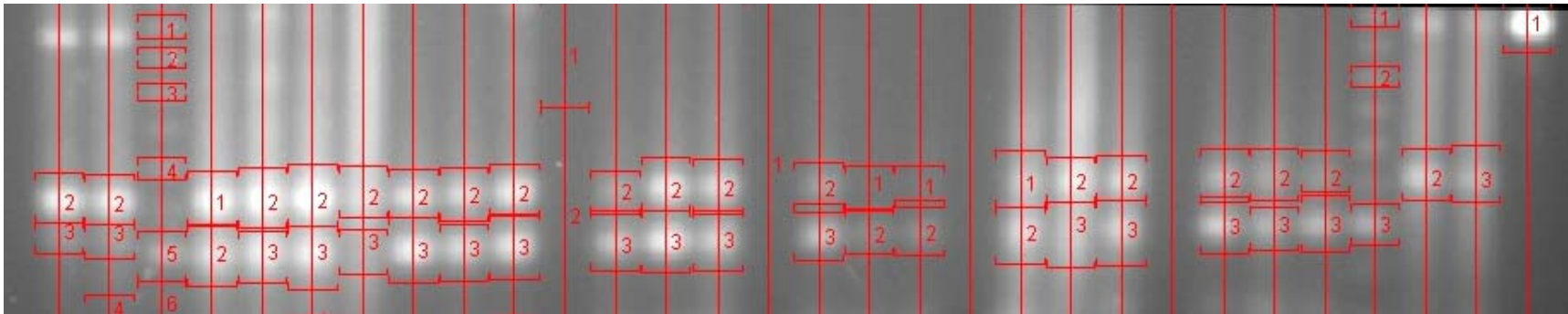
Y PCR Product: 61 gatatgagtggaaaaggcttacagatgctagaaaagcgcccattctttgaggaggcacag 120

SRY: 1365 agacta 1370

|||||

Y PCR Product: 121 agacta 126

Gel Picture



VITA

Tara M. Taylor was born on August 30, 1981 in West Monroe, Louisiana, to K. Terry Taylor and Lisa Spiller Taylor. She graduated from Ruston High School, Ruston, Louisiana, in 1999, where she participated in band and many FFA teams.

She then attended Southern Arkansas University in Magnolia, Arkansas to earn her Bachelor of Science degree in Agriculture Science. She especially enjoyed working on the SAU farm throughout college and graduated in August, 2003.

Tara began graduate studies at Louisiana State University in August, 2003, where she studied male reproductive physiology under Dr. John E. Chandler at the LSU Dairy Improvement Center. She also earned a minor in Mass Communication with a focus on print journalism. Within her time at LSU, she completed an internship at the Louisiana Cattlemen's Association as an editorial assistant. Tara will receive her Master of Science and minor in Mass Communication in August, 2005.

Tara plans to continue working in the field of agriculture after graduation.