PROPORTIONAL ASSESSMENT OF X AND Y CHROMOSOME-BEARING SPERMATOZOA IN BULL AND BOAR EJACULATES USING CONVENTIONAL AND REAL-TIME PCR TECHNIQUES

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ABSTRACT

Considerable variation in the percentage of Y-chromosome bearing spermatozoa (%Y-CBS) between ejaculates in the same male has been reported. Collection regime has been speculated to influence the degree of variation in %Y-CBS in bull ejaculates (Chandler et al., 1998). Experiments were designed to evaluate the effects of two collection regimes on %Y-CBS. Conventional PCR combined with gel electrophoresis and image analysis was employed to determine %Y-CBS in ejaculates from two bulls collected on 7-day intervals and two bulls on 21-day intervals. Real-time PCR technology was used to quantify %Y-CBS in the same ejaculates. Boar ejaculates were also analyzed with both techniques and compared to the percent male piglets in litters resulting from the assayed ejaculates. Collection day ($P<0.0001$) significantly affected %Y-CBS as determined by both PCR methods. Ejaculate nested within bull ($P<0.07$) was significant in the conventional PCR study and collection frequency ($P<0.0001$) in the realtime PCR trial. Ejaculate nested within boar was highly significant ($P<0.0001$) for both technologies. Boar was significant ($P<0.002$) in the conventional PCR study. Predicted %Y-CBS determined by real-time PCR was significantly correlated (0.52, $P=0.004$) to percent male piglets. Both PCR techniques were effective in quantifying the variation in %Y-CBS in bull and boar ejaculates. Manipulation of %Y-CBS via collection regime may prove valuable for altering the secondary sex ratio in animal agriculture.

CHAPTER 1

INTRODUCTION

Controlling the gender of progeny, in both humans and production animals, has been of great interest for decades. Predetermination of sex in human offspring would be of great value in preventing the occurrence of sex-linked diseases when medically indicated. Effective, reliable methods of sex predetermination would also allow family balancing when desired.

Gender selection in farm animals has great economic advantages. Preselection for females in the dairy industry would reduce the losses in both income and genetic progress incurred by the producer with the birth of each bull calf. The swine industry would benefit in both the market and seedstock sectors if the pre-selection of piglet gender was available. Selection for gilt pigs would allow for greater efficiency in the production of prospective brood sows by contract breeders in integrated production schemes. The efficiency of production and the quality of pork for consumption could be improved through gender selection due to the tendency of gilts to product leaner, more efficient, and thus more valuable, carcasses. Likewise, the greater growth rate of beef steers over heifers generates a desire to alter the sex ratio toward males in market beef operations.

Many techniques of the past three decades have focused on the physical separation of X- and Y-bearing spermatozoa. These techniques have been largely unsuccessful, with the exception of flow cytometric cell sorting. However, even this procedure has yet to yield sorted semen in large enough quantities or of adequate quality to be used in conventional commercial artificial insemination.

The scientific literature offers evidence that natural variation in sex chromosome content exists between ejaculates in humans and cattle. Several techniques have been employed for measurement of this variation. The polymerase chain reaction has been shown to be an accurate tool for estimation of sex chromosome composition of semen. The use of PCR as a semen assay for the manipulation of secondary sex ratio would be of great benefit to animal agriculture, as well as human reproductive medicine.

CHAPTER 2

REVIEW OF LITERATURE

SEX RATIO

Nalbandov (Nalbandov, 1964) defined three types of sex ratios. The primary sex ratio is determined by the ratio of male to female zygotes. The secondary sex ratio is proportion of male to female offspring at birth, while the tertiary sex ratio is comprised of the numbers of male versus female offspring reaching puberty. The expected ratio in a large population of offspring is 1:1, but has been shown to vary significantly (Nalbandov, 1964).

Most study of sex ratios has involved observation of secondary sex ratios and subsequent attempts to explain unexpected variations. Bernstein (Bernstein, 1954) reported an effect of paternal occupation on secondary sex ratio in humans. James (1994b) found that occupational exposure of men to deleterious environments was associated with fathering excesses of daughters. Khoury et al. (1984) documented a racial effect on sex ratio in humans where white couples produced higher proportions of boys than black couples in the United States. It was speculated from information gathered via interracial crosses that differences were due to the fathers' and not the mothers' race.

Men suffering from non-Hodgkin lymphoma (Olsson and Brandt, 1982), and multiple sclerosis (James, 1994a) are reported to father a preponderance of daughters, while men with prostatic cancer (James, 1990) and hepatitis B (Chahnazarian et al., 1988) father more sons.

Animal sex ratio studies have produced a variety of hypotheses on the causative mechanisms behind skewed secondary sex ratios. Lambin (1994) found that the sex ratio in litters of Townsend's voles varied with animal population density. When vole numbers were high, the secondary sex ratio was 1:1. In years of low density, nearly 70% of pups born were female. It was postulated that increased availability of food and space led to increased cooperation between animals and created favorable conditions for predominantly female litters. Berger et al. (1987) documented an effect of the plant metabolite 6-methoxybenzolinone on sex ratio and breeding performance in montane voles.

Arnbon et al. (1994) related sex ratio in elephant seal pups to maternal size. Females in a smaller size group gave birth to only female pups. This was considered to be an evolutionary adaptation to the additional energy requirements placed on the cow seal by male pups resulting in offspring gender being determined by the overall size or energy reserve of the female.

The secondary sex ratio of ungulate species was tightly associated with food supply (Hoefs and Nowland, 1994). Evaluation of six captive species revealed that more females were born than males, purportedly because of the high level of nutrition available. This conclusion was drawn on the observation that wild animals regulate herd size to the food supply by producing more males in lean years and more females in years of plenty (Hoefs and Nowland, 1994).

Great debate has been waged over the possible causative mechanisms for secondary sex ratio variation in swine (Meikle et al., 1993; Mendl et al., 1997; James,

1997; James, 1998; Mendl et al., 1998; Meikle et al., 1998). James (1996) proposed an effect of parental hormone levels at the time of conception on litter sex ratios in pigs. Rodent studies have shown that females that gestate between two males are more androgenized postnatally than females that gestate between females. This phenomenon and differences in the sex ratio of offspring from the two groups of females has been demonstrated in the Mongolian gerbil (Clark et al., 1993; Clark and Galef, 1995) and house mouse (Vandenbergh and Huggett, 1994).

Many factors have been purported to alter the human secondary sex ratio. Andersson and Bergstrom (1998) related low birth ratio to short stature and obesity, both of which are influenced by maternal nutritional state. Increased secondary sex ratio has been associated with increasing age gap between parents (Astolfi and Zonta, 1999). A significant skew toward male births was found in a subsample with 16-25 years difference in parental age, but no overall effect of age gap was found when all births in the two year study were included. Nonaka et al. (1999), using a historical database, found a lowered sex ratio in the months of February-April and May-July in a French Canadian population. The season of the mother's birth was also claimed to affect the sex ratio of her children. Mothers born in February-April bore fewer male children while the father's birth season had no effect. Studies of this nature are often retrospective in nature, and thus, subject to bias, and should be evaluated with the proper measure of caution.

SEX SELECTION AND SPERM SEPARATION

Attempts to alter the sex ratio have been focused in three predominant areas: 1) in vivo clinical manipulation of timing of breeding , ovulation and insemination, 3) in vitro

sperm separation techniques, and 2) preimplantation diagnosis (Reubinoff and Schenker, 1996).

Many of the earlier reports on sex preselection studied the effects of timing of coitus relative to ovulation. The underlying basis for these studies was the theory that Y-bearing spermatozoa swim faster but do not survive as long as X-bearing spermatozoa. Thus, coitus well before ovulation was theorized to produce a preponderance of male births and coitus during or after ovulation would result in more female offspring. Contradicting this theory, Kleegman (1954) reported in humans that insemination hours before ovulation resulted in a significantly higher percentage of males, while insemination days before ovulation significantly increased the number of females.

Shettles (1970) reported a greater number of human males (86%) in cases where conception occurred at midcycle while conceptions more distant from ovulation resulted in more females (84%). Greater numbers of male infants with midcycle conceptions was also reported by Billings and Westmore (1982). Other studies were, however, unable to confirm these findings (Simcock, 1985; France et al., 1992). Furthermore, contradictory studies exist reporting female predominance in midcycle conceptions and male predominance earlier or later in the cycle(Guerrero, 1974; Harlap, 1979).

In a review of 12 reports, Zarutskie (1989) examined the effect of ovulation induction and artificial insemination on sex ratio in human assisted reproduction. Although there was great variety in protocols, semen sources, ovulation induction medications, and insemination techniques a statistically significant skew in the sex ratio toward males (7-10% increase) occurred when artificial insemination was performed. The lack of congruency among the studies reviewed and the only slight increase in male

births diminish the practical importance of ovulation induction and artificial insemination for the alteration of sex ratio. The only thing that is clear about in vivo clinical manipulation of sex ratio is that none of the purported techniques give reproducible results and much skepticism exists about their efficacy.

Preimplantation sex determination provides more definitive selection techniques for controlling the sex of offspring. Current techniques include PCR amplification of DNA from biopsied blastomeres or cytogenic preparation of single blastomeres for FISH. The first clinical studies incorporated PCR amplified Y-specific sequence in order to identify male embryos (Handyside et al., 1990). These studies led to successful transfer of female embryos to mothers who were carriers of serious X-linked disorders. Since then studies have incorporated simultaneous amplification of different and homologous X- and Y-specific sequences to prevent misclassification due to amplification failure (Nakahori et al., 1991; Grifo et al., 1992).

Dual FISH with different colors for simultaneous detection of X- and Y-specific sequences has been used to select embryos for sex (Griffin et al., 1992). Some advantages of FISH over PCR are its ability to identify hyperploid embryos, low incidence of contamination of the assay by foreign DNA and its relative speed in assessment of embryo gender.

Since Ericsson et al. (Ericsson et al., 1973) reported enrichment of Y-bearing spermatozoa to 85% after passage through discontinuous albumin gradients, many procedures have been designed to separate sperm populations into X and Y fractions using gradient separation. The methods have included albumin gradients (Ericsson et al., 1973; Ross et al., 1975; Quinlivan et al., 1982; Brandiff et al., 1986; Welch et al., 1995;

Claassens et al., 1995; Flaherty et al., 1997), Percoll gradients (Kaneko et al., 1983 #287; Iizuka, 1987 #195; van Kooij, 1992 #217; Wang, 1994 #220] and Sephadex columns (Steeno et al., 1975; Adimoelja et al., 1977; Schilling and Thormaehlen, 1977).

Controversy has long existed over the use of discontinuous albumin gradients to enrich X- or Y-bearing human sperm populations. The underlying theory of gradient separation of sperm is the assumption that X- and Y-bearing sperm differ in their motility and longevity, with the Y-sperm are smaller and mover faster but are shorter lived. Thus, Ericsson et al. (Ericsson et al., 1973) claimed the apparent enrichment of Y-sperm was due to their faster movement through the viscous albumin. Several conflicting reports (Ross et al., 1975; Evans et al., 1975; Brandiff et al., 1986; Ueda and Yanagimachi, 1987) have been published since. Many studies relied on data from clinical trials lacking control groups and unbiased evaluation (Dmowski et al., 1979; Beernink and Ericsson, 1982; Corson et al., 1983; Corson et al., 1984; Beernink et al., 1993). Perhaps the most unreliable characteristic of early work was the use of quinacrine staining to classify sperm cells (Barlow and Vosa, 1970; van Kooij and van Oost, 1992). More recent reports comparing quinacrine staining with modern molecular techniques found quinacrine staining to be unreliable due to staining inefficiency (van Kooij and van Oost, 1992). More reliable techniques have been used to evaluate the true chromosomal content of sperm cells after passage through albumin gradients. Wang et al. (1994), Classens et al. (1995) and Flaherty et al. (1997) used FISH to evaluate separation efficiency. Either no change in X:Y ratio was found (Wang et al., 1994; Flaherty et al., 1997) or differences, though statistically significant, were too small to be clinically practical (Claassens et al., 1995).

Kaneko et al. (1983) reported enrichment of X-bearing sperm to 83% using discontinuous 6-step Percoll gradients. Iizuka et al. (1987) used 8- and 12-step Percoll gradients and reported 82% and 94% X-enrichment respectively. Both studies scored separation fractions using quinacrine staining.

Wang et al. (1994) scored cells passed through a 12-step Percoll gradient using dual FISH and found only a 5% enrichment of X-bearing sperm. van Kooij and van Oost (1992) employed Southern blot analysis and DNA probes to evaluate separation on 12-step Percoll gradients The X:Y ratio as found to be 1:1. These authors also performed quinacrine staining on the same samples and found that 86% of sperm did not exhibit a signal. This failure to stain would result in a high percentage of false positives for X-bearing spermatazoa.

Reports of successful enrichment of X-bearing sperm using Sephadex column filtration (Steeno et al., 1975; Adimoelja et al., 1977; Schilling and Thormaehlen, 1977; Quinlivan et al., 1982; Corson et al., 1983) met the same fate as albumin and Percoll studies. All of these studies used quinacrine staining to evaluate separation and were later shown to be inaccurate by studies using DNA probes (Beckett et al., 1989), FISH (Vidal et al., 1993) and PCR (Lobel et al., 1993).

Swim-up procedures have been purported to separate X- and Y-bearing sperm based on their relative motility. Standard swim-up procedures yielded either no enrichment (Engelmann et al., 1989) or clinically insignificant Y-enrichment (Claassens et al., 1989). Swim-up procedures specifically modified to select sperm have been applied. Check and Katsoff (1993) reported 88.5% male offspring with modified swim-up compared to 50% on standard Percoll gradients. Check (1989) also reported 81%

male offspring in a separate study using modified swim-up. This study also classified the semen in samples using quinacrine staining. Subsequent studies incorporating FISH (Han et al., 1993; De Jonge et al., 1997) and PCR (Lobel et al., 1993) showed no enrichment beyond a 1-2% Y-enrichment (De Jonge et al., 1997) on Sephadex columns.

Perhaps the most promising method to date for separation of X-and Y-bearing spermatozoa is sorting by flow cytometry. Mammalian sperm exhibit differences in DNA content due to size differences in the X and Y chromosomes, ranging from 2.8% in the human to 12.5% in the creeping vole. Most agricultural production species (cattle, swine, etc.) exhibit a 3-4% difference in DNA content with the X-chromosome bearing spermatozoa containing the greater amount of genetic material. This difference can be exploited by staining the spermatozoal DNA with a fluorescent dye and sorting via flow cytometry based on relative fluorescent intensity (Gledhill, 1988). The basis of flow cytometric sperm sorting is described in detail in Johnson (Johnson and Schulman, 1994). This technique has been applied to both isolated sperm nuclei (Johnson et al., 1987a; Johnson et al., 1987b) and live sperm (Johnson et al., 1989; Johnson et al., 1993) with purities of over 90%. The degree to which sperm can be separated is quite species-specific and varies from animal to animal and sample to sample.

Flow cytometrically sorted (FCS) sperm have been used to successfully produce offspring in several species. Offspring resulting from surgical insemination of sorted sperm have been obtained in rabbits (Johnson et al., 1989) and swine (Johnson, 1991). In vitro fertilization with sorted sperm have yielded young in cattle (Cran et al., 1993; Cran et al., 1995) and swine (Rath et al., 1997). Intracytoplasmic sperm injection resulted in the birth of a lamb (Catt et al., 1996) and calves (Hamano et al., 1999) from sorted sperm.

Cattle (Seidel et al., 1997) and sheep (Cran et al., 1997) pregnancies and births were recently reported after nonsurgical (deep uterine) artificial insemination with low numbers (100,000) of sorted sperm. Secondary sex ratios in all of these studies agreed with the predicted outcomes based on estimated primary sex ratios determined by reprocessing sorted samples to quantify separation efficiency.

Although successful use of sorted sperm has been demonstrated, the FCS method has several drawbacks. The predominant shortcoming of FCS for gender preselection is the low yield of sorted sperm (2-3 x 10^5 sperm/hour), thus limiting its use to IVF and ICSI applications (Flaherty and Matthews, 1998). The standard insemination dose for cattle of 10-15 million sperm would require days to produce via FCS. In addition, the compromised viability of sorted sperm, as indicated by delayed embryonic development and reduced pregnancy rates (Johnson, 1997), further reduces the usefulness of FCS sperm for commercial AI. Concerns have been expressed about the safety of using fluorochrome stains and ultraviolet illumination, both of which are potentially mutagenic, in flow sorting (Ashwood-Smith, 1994; Johnson and Schulman, 1994).

The polymerase chain reaction has been used to determine embryonic sex in cattle (Peura et al., 1991), pigs (Fajfar-Whetstone et al., 1993; Pomp et al., 1995), humans (Handyside et al., 1989) and mice (Kunieda et al., 1992). Advantages of PCR sex determination are that it is simple, inexpensive, rapid, and maintains a high degree of accuracy.

Amplification of a Y-specific sequence from embryo DNA has been used to identify male embryos (Handyside et al., 1989; Handyside et al., 1990; Han et al., 1993; Bradbury et al., 1990). Other studies have incorporated an autosomal or X-specific

positive control for the PCR assay (Peura et al., 1991; Kunieda et al., 1992; Avery et al., 1992; Wu, 1993; Kirkpatrick and Monson, 1993; Valdivia et al., 1993; Pomp et al., 1995). The inclusion of such controls greatly decreases the incidence of female false positives due to amplification failure. Duplex PCR gender assays have been used to identify the chromosome composition of spermatozoa in single cell sorts (Welch et al., 1995).

The nature of PCR sexing assays is almost entirely qualitative due to the limited amount of template for amplification, i.e. DNA from an embryo biopsy or a single sperm cell. However, if careful attention is paid to the dynamics of the PCR reaction and template preparation, it can become a quantitative assay with at least one valuable application.

Lobel (1993) described a quantitative PCR assay for determining the primary sex ratio in human semen. Known amounts of extracted and purified sperm DNA template were placed into duplex PCR reactions. The triplicate reactions co-amplified homologous X and Y chromosome-specific alleles in the presence of a radiolabled dNTPs. The PCR products were resolved by restriction endonuclease digestion and polyacrylamide gel electrophonesis. Autoradiography of dried PAGE gels was performed and densitometry of autoradiograms was achieved by optical scanning. The ratio of X to Y sex chromosome-specific signal was converted, by reference to control male DNA ratios from the same experiment, to a percentage of amplified sex chromosome sequences that were of Y chromosome origin. Results yielded a normal distribution of Y percentages with a mean very close to 50% (range 41.91 to 56.77%Y). Triplicate

measures of each sample clustered tightly about the sample means indicating a high degree of precision in the assay.

The polymerase chain reaction has been used to evaluate primary sex ratio bull semen (Chandler et al., 1998). PCR was performed on a constant amount of sperm DNA and amplification product electrophoresed on agarose gels stained with ethidium bromide. Stained gels were illuminated with UV light and images of fluorescing bands captured with a SIT camera and a JAVA-enabled frame grabber. Relative intensities of bands were calculated via image processing software and compared to a pooled semen DNA standard. Intensity differences between bands from individual ejaculates estimated relative percent Y chromosome-bearing sperm content of each ejaculate. When all known sources of variation were accounted for statistically, the percentage of sperm bearing the Y chromosome per ejaculate ranged from 24" 9.8 % to 84" 9.8 % with an overall mean of 50.0" 4.6 %. Twenty percent of all ejaculated differed significantly from the overall mean.

CONVENTIONAL PCR

The polymerase chain reaction was developed by scientists at the Cetus Corporation (Saiki et al., 1988). Technologies and techniques based on PCR offer great power in the detection and analysis of DNA and RNA. As little as a single copy of a particular sequence can be amplified in a highly specific manner for sequence detection or analysis. Applications of this robust method include DNA sequencing, study of gene expression, forensic analysis, identification of organisms, mutations and genetic defects among numerous others (Erlich et al., 1991; Erlich and Arnheim, 1992).

REAL-TIME PCR

Higuchi et al. (1992, 1993) developed a system that detects PCR products as they accumulate in the reaction tube. The inclusion of the intercalating fluorescent dye, ethidium bromide (EtBr), in the reaction tube allowed the visualization and crude quantitation of increasing concentrations of double-stranded DNA (dsDNA) as amplification progressed. Quantitation of dsDNA was initially achieved by directing ultra-violet (UV) radiation through the walls of the amplification vessels with a transilluminator and photographing with a red filter (Higuchi et al., 1992). This measurement technique only allowed for relative quantitation of DNA amounts. Improvements to the measurement system were implemented by epoxying a bifurcated optical fiber into the mouth of a reaction tube in order to deliver excitation illumination to the tube and return EtBr fluorescence to a spectrofluorometer for measurement (Higuchi et al., 1992). Accurate measurement of fluorescent intensity with the fluorometer enabled the researchers to achieve absolute quantitation of dsDNA in the PCR tube after each cycle. Monitoring of multiple PCRs was later achieved by recording fluorescence of all tubes in a thermocycler block with a video camera after each cycle (Higuchi et al., 1993). The average intensity of emitted light from each tube was obtained using image analysis software yielding a characteristic plot for each reaction. Fluorescence detection systems have since been improved with the adaptation of laser excitation via fiber optics and spectral resolution of fluorescent light with charge-coupled device (CCD) arrays.

The principle drawback to intercalator-based PCR product detection is that both specific and nonspecific products generate signal. The development of an assay

exploiting the 5' nuclease activity of *Taq* DNA polymerase (Holland et al., 1991) provided an elegant method for detecting only specific amplification products. Specificity was achieved by the addition of a probe labeled with ^{32}P at the 5' end and blocked at the 3' end to prevent it from acting as a primer. During amplification, the annealed probe was cleaved by the polymerase only as it extends from an upstream primer. Following PCR, thin layer chromatography was used to separate cleaved from intact probe.

Lee et al. (1993) completely eliminated post-PCR processing in real-time reactions. Fluorogenic oligonucleotide probes with both a reporter dye attached to the 5' end and a quencher dye on the 3' end (Livak et al., 1995) were added to the reaction. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter due to Förster resonance energy transfer (FRET) through space. During amplification, if the target sequence is present, the probe anneals downstream from one of two flanking primers. As the primer is extended, the probe is cleaved by the 5' nuclease activity of *Taq* DNA polymerase and the reporter dye is separated from the quencher resulting in an increase in fluorescent signal from the reporter. The inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from probe with each PCR cycle resulting in an increasing amount of fluorescent signal proportional to the growing amount of product.

The advantages of fluorogenic probes over intercalating or minor groove-binding dyes are that specific hybridization of the probe to the target sequence and successful primer extension are required for the generation of signal. These requirements eliminate

the potential quantitative errors associated with mispriming or primer-dimer formation. Another advantage is realized when advanced CCD arrays are used for spectral resolution of the fluorescent light, allowing the use of two probes with different dyes to detect amplification of two distinct target sequences in the same PCR reaction.

Quantitation analysis of data obtained from a real-time PCR instrument is accomplished by detecting the point during thermocycling when amplification of a PCR product is first detected rather than the amount of product accumulated after a fixed number of cycles. An amplification plot is created by plotting fluorescent signal (Y-axis) versus cycle number (X-axis). There is minimal change in fluorescent signal in the first few PCR cycles and this establishes a baseline for the amplification plot. Any increase in signal above the baseline is due to amplification of the target sequence. When the amount of signal passes a user-defined threshold a C_T (threshold cycle) is established. The C_T is defined as the fractional cycle number at which the fluorescence passes a fixed threshold (Higuchi et al., 1993). A plot of the log of initial target copy number for a set of standards versus C_T is a straight line. Thus, quantitation of the amount of target in unknown samples is achieved by determining their C_Ts and using the standard curve to estimate starting copy number. The use of C_T values, instead of endpoint measurements, greatly increases the dynamic range of quantitation because data is collected for every PCR cycle, ensuring that measurements are not taken after amplification has reached a plateau.

CHAPTER 3

ASSESSMENT OF SEX CHROMOSOME COMPOSITION OF BULL EJACULATES USING CONVENTIONAL AND REAL-TIME PCR TECHNIQUES

INTRODUCTION

Considerable variation in the percentage of Y-chromosome bearing spermatozoa between ejaculates within the same male has been reported (Johnson, 1994; Chandler et al., 1998). The percentage of male offspring produced by insemination with single ejaculates demonstrates this further (Chandler et al., 1998). Variation in male offspring changed as the interval between semen collections changed (Chandler et al., 1998). Collection regime manipulation was suggested to maximize the variation (Chandler et al., 1998).

Early work showed that the total sperms produced in consecutive ejaculates decreased in a quadratic fashion (Almquist and Hale, 1956; Hafs et al., 1959). The total sperms per ejaculate decreased by 73% when comparing a collection frequency of once per week to once per day (Hafs and Boyd, 1971). When evaluated as total sperms collected per week, there was a 90% increase when bulls were collected once per day (Hafs and Boyd, 1971). Workers were making bull management recommendations to maximize sperm harvested per time spent harvesting. Regimes were varied to meet the objective of harvesting different kinds of spermatozoa, namely X- or Y-chromosome bearing sperm.

One function of the cauda epididymides is to store spermatozoa. The majority of extragonadal reserve exists in the cauda epididymis for both sexually rested bulls (54%) and those on a daily collection scheme (43%) (Amann and Almquist, 1976). Different collection regimes make a small change in the storage function of the cauda epididymis (Amann and Almquist, 1976). Another function may be sorting of the sperm based on head size. This may explain some of the differences in percent Y-chromosome bearing spermatozoa (%Y-CBS) from ejaculate to ejaculate and subsequent differences in percent male offspring obtained from single ejaculates (Johnson, 1994; Chandler et al., 1998).

Conventional PCR and gel electrophoresis techniques combined with image analysis have been employed to quantify %Y-CBS in bull ejaculates (Chandler et al., 1998). Recent advances in PCR techniques and instrumentation open opportunities to design assays with improved precision (Higuchi et al., 1993). With this in mind, an experiment was designed to evaluate the effect of different collection frequency regimes on the variation of percent %Y-CBS found in successive ejaculates within individual bulls using both conventional and real-time PCR technologies.

MATERIALS AND METHODS

Semen Collection and Processing

Four yearling Holstein bulls were sexually rested for 21 days before semen collection for the study commenced (reprinted from Theriogenology). The bulls were randomly assigned to be collected either once weekly or every 21 days for 105 days. Two ejaculates were collected from each bull on every collection day. Semen collection was achieved by subjecting the bulls to a sexual preparation period of two false mounts

separated by ten minutes of active restraint. On a third mount, the bulls were allowed to ejaculate into a water-jacketed artificial vagina (AV) maintained at 60EC. Semen was allowed to drain by gravity into an attached 15 ml conical tube which was immediately removed from the AV, capped and placed into a 37EC waterbath until evaluation. Sperm concentration of 1:80 diluted subsamples was determined using a spectrophotometer that had been calibrated with replicated hemacytometer counts regressed on optical density over a wide range of sperm densities. Semen was extended in a whole milk-glycerol extender with antibiotics to approximately 30 million spermatozoa per milliliter. Extended semen was packaged in 0.5 ml straws, frozen via a -19EC/min protocol (Chandler, 1984) and stored under liquid nitrogen.

DNA Extraction

One straw from each ejaculate was thawed and aliquots from each straw were combined to construct a pooled sample containing equal numbers of sperm from every ejaculate in the study. Each ejaculate and the pooled sample were extracted by a modified protocol of Aravindakshan et al. (1998). Samples were washed twice with 2.9% sodium citrate solution and resuspended in 50EC lysis solution (Aravindakshan, 1998) and incubated at 50EC for 30 minutes. Proteinase K (Qiagen Inc., Valencia, CA) (5Fl) was added to the solution and incubated at 50EC for at least 2 hours or overnight. One volume of phenol:chloroform was added and the tubes gently shaken every 3 minutes for 15 minutes at room temperature. The samples were then centrifuged at 15,500 X g for 3 minutes, the organic phase aspirated, and the phenol:chloroform step repeated. Two volumes of ice cold absolute ethanol were added to the remaining aqueous phase and

shaken to precipitate the DNA. Samples were centrifuged for 1 minute at 15,500 X g and the ethanol carefully decanted. DNA pellets were dried in a vacuum until clear and resuspended in sterile water by incubating at 37EC with gentle agitation until dissolved. Nucleic acid concentration was determined by spectrophotometry at 260/280 nm.

Conventional PCR

In a duplex PCR reaction, specific segments of the Y-chromosome (SRY, sex-determining region Y) and the X-chromosome (F9, clotting factor IX) DNA were amplified using Ready-To-Go PCR beads (Pharmacia-Biotech, Piscataway, NJ). Primers to these segments were designed to produce similar size products (SRY, 151 bp; F9, 122 bp) as well as having approximately the same melting and annealing temperatures. Each reaction tube contained the primer sets for both segments, 300 ng template and the standard components of the Ready-To-Go bead. A thermocylcer was programmed as follows: hot start at 95EC, 1 cycle (2 min @ 95EC, 1 min @ 51EC, 2 min @ 72EC), 29 cycles (1 min @ 95EC, 1 min @ 51EC, 1 min @ 72EC), 10 min @ 72EC.

A standard curve was constructed by amplifying each target sequence separately, separating the product from genomic DNA and PCR reaction components via gel electrophoresis and purifying the product bands using spin columns. Each product was quantified by spectrophotometry and serially diluted until a dilution was identified that, after amplification, resulted in a band of equal intensity to that produced by 300 ng pooled DNA under identical PCR conditions. This amount of product was equated to the number of copies of both target sequences present in 300 ng of spermatozoal DNA from a sample with a 1:1 X:Y ratio of sperm. Diluted X and Y products were mixed in 4:1, 3:2,

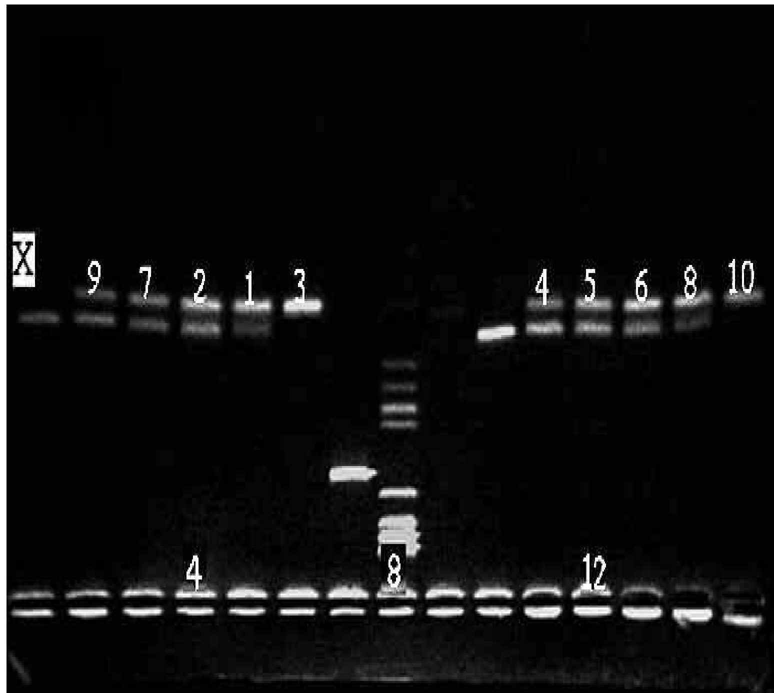
2:3 and 1:4 ratios to yield mixtures representing the 80:20, 60:40, 40:60 and 20:80 percent points, respectively, on a standard curve. Figure 3.1 depicts the standard curve mixtures electrophoresed in quadruplicate. These mixtures were amplified along with unknown samples in each PCR run and electrophoresed on the same gels. A lambda DNA control reaction supplied by the bead manufacturer and a blank reaction containing no DNA were included in each run as a controls for PCR failure and contamination of reaction components.

Electrophoresis

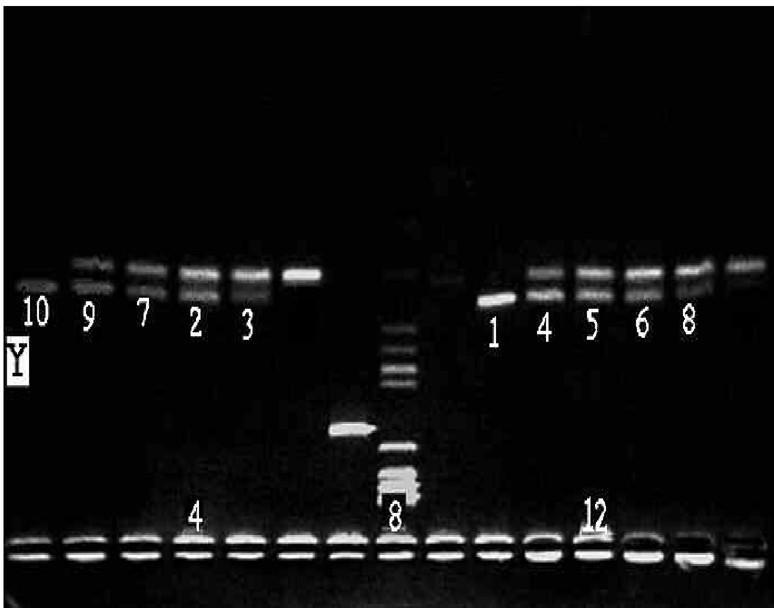
Amplified samples were electrophoresed on 4% agarose gels in 1X TAE buffer. One microliter of EtBr, for fluorescent staining of PCR products, was mixed into the molten agarose before pouring. Two 15 tooth combs were inserted into the molten agarose, dividing the gel into upper and lower sections. Two 11.25 Fl aliquots of PCR product from each tube were combined with 5 Fl loading buffer each. Each aliquot was placed in a well and electrophoresed for 1.5 hours at 127 VDC. Samples were placed on the gel in a crossing manner to eliminate any tube by gel position interactions. The upper section pattern was: lane 1, empty; lane 2, 20:80 standard; lane 3-6, unknown ejaculate samples; lane 7, lambda control; lane 8, DNA size marker; lane 9, blank; lane 10-13, unknown ejaculate samples; lane 14, 40:60 standard; lane 15, empty. The order was reversed on the lower gel section with the 60:40 and 80:20 standards in lanes 2 and 14, respectively.

Image Analysis

Images of EtBr stained gel were captured by a previously described method (Chandler et al., 1998). Image analysis was performed using UTHSCSA ImageTool



a.)



b.)

Lane	Sample Identity	X (a) object #	Y (b) object #
		Object # for each chromosome analysis	
1	0/100	-	10
2	20/80	9	9
3	40/60	7	7
4	60/40	2	2
5	80/20	1	3
6	100/0	3	-
7	Factory control		
8	DNA Ladder		
9	Negative control		
10	0/100	-	1
11	20/80	4	4
12	40/60	5	5
13	60/40	6	6
14	80/20	8	8
15	0/100	10	-

Figure 3.1. Agarose gel images of the double standard curve of DNA amplified from the clotting factor IX gene (a) on the X-chromosome and from the SRY gene (b) on the Y-chromosome and the ImageTool software object assignments.

(University of Texas Health Science Center at San Antonio, San Antonio, TX) software. Captured images were background subtracted before analysis. The boundaries of each band were identified by the software on each image using a consistent grey level threshold and were assigned object numbers. Integrated density (INGDEN), which is defined as the product of the average grey level and the number of pixels, was calculated by the software for each band.

Real-time PCR

Oligonucleotide primer pairs and fluorogenic probes were designed for the SRY and F9 genes using Primer Express software (Applied Biosystems Inc., Foster City, CA). To facilitate comparison of PCR techniques, target sequences for the real-time assay were designed to overlap as much as possible with those in the conventional PCR assay. Due to the design constraints imposed by the software, in order to produce primer/probe combinations for a standard thermocycler program, target sequence homology between assays was not 100% but was maintained above 50% for both targets. The advantage of designing probes and primers together for predetermined PCR conditions is that the efficiencies of both reactions will be very similar, yielding greater quantitative precision. This is especially important in duplex reactions where a highly efficient reaction could starve a less efficient one for reaction components.

Duplex reaction tubes for unknowns contained 100 ng spermatozoal DNA, 25 Fl PCR Master Mix (Applied Biosystems Inc., Foster City, CA), 900 nM each primer and 300 nM probe, q.s. sterile water to a total volume of 50 Fl. All reactions were amplified in duplicate tubes.

A standard curve was constructed by amplifying 20, 60, 100, 140 and 180 ng of DNA from an equal sperm number pooled sample. The 100 ng reaction represented a 50% X, 50% Y center point for the curve. The additional curve points represented 10, 30, 70 and 90% for each chromosome. This method was chosen because the unknown sample C_T's would be centered on the standard curve by design, eliminating the need for adjustment factors. Standard curve reactions were amplified in triplicate along with unknown samples on each plate.

Statistical Analysis

For the conventional PCR assay, the log of the ratio (LOGRATIO) of X INGDEN versus Y INGDEN was calculated for each standard curve point estimate. An inverse regression analysis (Ryan, 1997) of the 36 curve point estimates was used to obtain prediction equations for calculating sex ratio in each ejaculate. The X INGDEN for each observation was adjusted to the standard curve by multiplying by the ratio of the average ejaculate X INGDEN to the average X INGDEN of the standard curve. The same correction was applied to the Y INGDEN. Predicted values for ejaculates were analyzed using the Mixed Model option of the General Linear Model procedure in the SAS statistical package, Version 8 (SAS User's Guide: Statistics, 1999). Ejaculate source of variation was tested with an adjusted error term. Other effects in the model were tested with random error. Least squares means are presented where significance occurred. The model included the following:

$$Y_{ijklmn} = \text{F} + \text{Gel Position}_i + \text{PCR Tube}_j + \text{Collection Frequency}_k + \text{Bull(Collection Frequency)}_{l(k)} + \text{Ejaculate(Collection Frequency*Bull)}_{m(lk)} + \text{Collection Day(CollectionFrequency*Bull*Ejaculate)}_{n(mlk)} + e_{ijklmn}$$

where:

Y_{ijklmn} = predicted percent Y-chromosome bearing spermatozoa

F = overall mean

Gel $Position_i$ = fixed effect of gel position i

PCR $Tube_j$ = fixed effect of PCR tube j

Collection $Frequency_k$ = fixed effect of collection frequency k

Bull(Collection $Frequency)_{l(k)}$ = random effect of the lth bull nested within k^{th} collection frequency

Ejaculate(Collection Frequency$(Bull)_{m(lk)}$ = random effect of the mth ejaculate nested within the interaction of the lth bull and the kth collection frequency

Collection Day(Collectio Frequency* Bull*$Ejaculate)_{n(mlk)}$ = random effect of the nth collection day nested within the interaction of the mth ejaculate and the lth bull and the kth collection frequency

e_{ijklmn} = random error

For the real-time PCR assay, the log of the ratios of X C_T versus Y C_T was calculated for each standard curve point estimate. Inverse regression of standard curve points and analysis of predicted values were performed as in the conventional PCR assay. Ejaculate source of variation was tested with an adjusted error term. Other effects in the model were tested with random error. Least squares means are presented where significance occurred. The model included the following:

Y_{ijkl} = F + Collection $Frequency_i$ + Bull(Collection $Frequency)_{j(i)}$ + Ejaculate$(Freq*Bull)_{k(ij)}$ + Collection Day(Collection Frequency* Bull*Ejaculate$*)_{l(ijk)}$ + $Plate_{ijkl}$ + e_{ijkl}

where:

Y_{ijkl} = predicted percent Y-chromosome bearing spermatozoa

F = overall mean

$\text{Collection Frequency}_i$ = fixed effect of collection frequency i

$\text{Bull(Freq)}_{i(j)}$ = random effect of the jth bull nested within the ith collection frequency

$\text{Ejaculate(Freq*Bull)}_{k(ij)}$ = random effect of the kth ejaculate nested within the interaction of the ith collection frequency and the j^{th}bull

$\text{Collection Day(Collection Frequency*Bull*Ejaculate)}_{l(ijk)}$ = random effect of the lth collection day nested within the interaction of the ith collection frequency and the jth bull and the lth ejaculate

Plate_{ijkl} = linear covariate to account for differences across plates used in PCR runs

e_{ijkl} = random error

RESULTS AND DISCUSSION

Conventional PCR

Figure 3.2 illustrates the standard curve produced by amplifying proportionately mixed PCR product. The regression was highly significant ($P<0.0001$) with R^2 values of 85% and 84% for the regression of LOGRATIO on %Y-CBS and %X-CBS, respectively.

Table 3.1 contains the analysis of variance for the applied model and Table 3.2 lists the expected mean squares used for appropriate tests for each source in the model. The method of applying the PCR product in a crossing design eliminated the possibility of a significant PCR tube by gel position interaction. The significant PCR tube effect

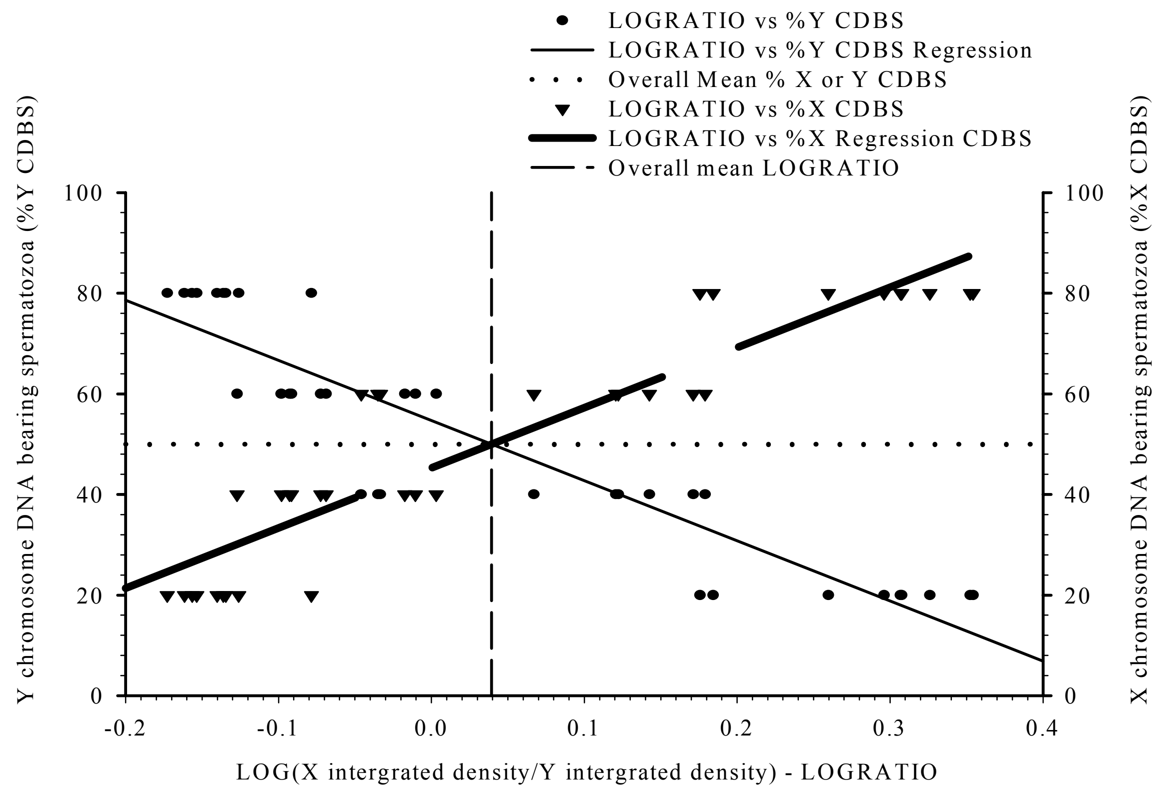


Figure 3.2. Duplex standard curve showing the relationship between the percentage X- and Y-bearing spermatozoa and the log ratio of PCR product integrated densities.

could result from different thicknesses within the gel and placing the replicate products on outside (lane 2) and center lanes (lane 10). The gel position effect resulted from a similar effect. This could be due to electrical resistance exerted by the lower gel section

Table 3.1. Analysis of variance table for the effects of gel position, PCR tube, collection frequency and appropriate interactions.

Source	df	MS	F-Value	Pr < F
Model	76	119.58	2.28	<0.0001
Gel position	1	232.05	4.43	0.0366
PCR tube	1	271.25	5.18	0.0238
Collection frequency (Freq)	1	317.47	2.96	0.0896
Bull(Freq)	2	190.28	1.76	0.1799
Ejaculate(Freq*Bull)	4	239.95	2.20	0.0773
Collection day(Freq*Bull* Ejaculate)	67	112.65	2.15	<0.0001
Error = MS(error)	217	52.44		
Corrected total	293			
R^2 (%)	44			

Table 3.2. Type III expected mean squares used for hypothesis testing.

Source	Type III Expected Mean Square
Gel position	Var(Error) + Q(Gel Position)
PCR tube	Var(Error) + Q(PCR Tube)
Collection Frequency (Freq)	0.9082 x MS(Collection Day(Freq*Bull*Ejaculate)) + 0.0918 x MS(Error)
Bull*(Freq)	0.9649 x MS(Collection Day(Freq*Bull*Ejaculate)) + 0.0351 x MS(Error)
Ejaculate(Bull*Freq)	0.9066 x MS(Collection Day(Freq*Bull*Ejaculate)) + 0.0392 x MS(Error)
Collection Day (Ejaculate*Bull* Freq)	MS(Error)

before the electromotive forces can act on the DNA in the wells of the upper section. There were significant contributions to the variation in %Y-CBS by ejaculate within bull by frequency treatment and collection day within bull by ejaculate by collection frequency treatment.

The overall response of %Y-CBS in ejaculates collected from bulls on a 7-day schedule fluctuated in a sinusoidal manner with a period of approximately 13 days. The 32% of the ejaculates were beyond one standard deviation of the overall mean (55%). Seven of the ejaculates (12.5%) were discernibly different from other ejaculates collected on the same day.

As seen in Figure 3.3, there was an oscillation in the average %Y-CBS across the entire experiment. Non-linear regression techniques were used to discern the pattern of this fluctuation. It was noted that the pattern modulated at day 63 and then repeated. Therefore, the non-linear regression was run on the first 63 days and then from day 63. In both segments of the prediction function, the period was established to be approximately 13.5 days. Within the first 63 days, there were 4.2 waves, afterwards there were 2.4 waves with equivalent periods. The amplitude of the waves prior to 63 days was greater than that afterwards. This non-linear regression analysis yielded data that was very similar to certain known constants associated with spermatogenesis in the bull. For example, the amount of time between initiations of spermatogenic cell generations is 13.5 days and is known as the length of the spermatogenic cycle (Johnson, 1994). Approximately four 13.5 day periods are necessary to complete the formation of a spermatozoa from a spermatogonium for a total of 61 days (Johnson, 1994). While the prediction functions did not fit the data curve exactly, the non-linear solutions gave

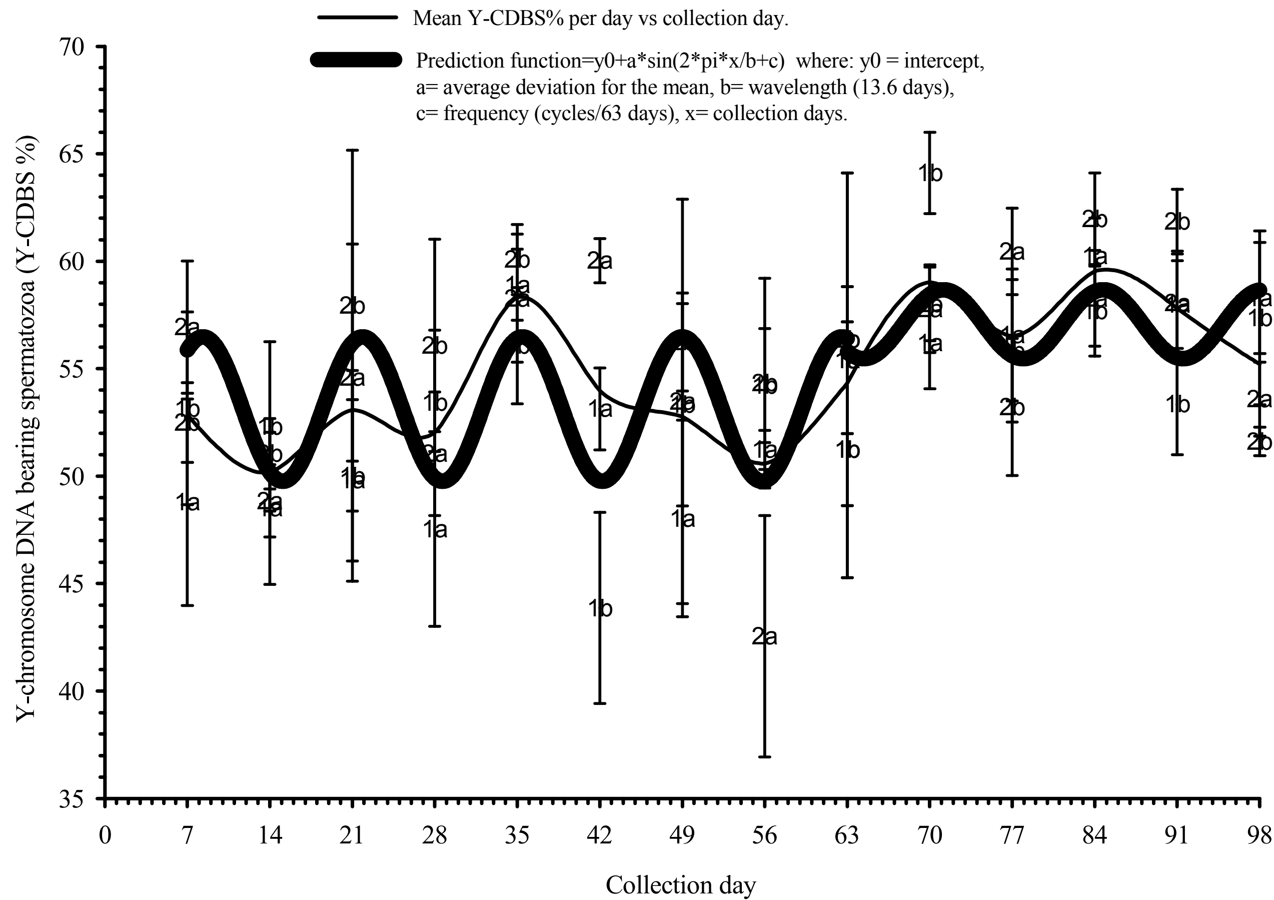


Figure 3.3. Percent Y-chromosome bearing spermatozoa as affected by 7-day collection frequency. Symbols represent mean %Y-CBS for ejaculates (a, b) from each bull (1, 2) on each collection day.

reasonable estimates of these known constants, i.e. a 13.5 -day period and 4.2 cycles of this period to give 63 days before a change is seen with a subsequent resumption of the process at the same frequency. Exactly how the fluctuation in %Y-CBS was influenced by spermatogenesis is not known but warrants further investigation.

A periodic overall response was also seen in %Y-CBS for ejaculates collected from bulls on a 21-day schedule (Figure 3.4). Of the 10 ejaculates taken from bull 3, three of them were beyond one standard deviation of the overall 21-day mean schedule of 63.4%, while two of these three were different fro the other ejaculates taken that day. Ejaculates taken from bull 4 were very similar on a within day basis, yet were still periodic across the 105 days of collection.

The cyclic nature of the responses from bulls collected both on the 7- and 21- day collection schemes could imply some testicular function. This is reminiscent of the cyclic nature of spermiogenesis, i.e. the time from the formation of the primary spermatocyte to spermiation, reported to be approximately 43 days. Thermal insult will cause cyclic perturbations in spermiogenesis which appear as increases in different types of spermatozoal abnormalities (Johnson, 1994).

Real-time PCR

Figure 3.5 shows the standard curve used in prediction of %Y-CBS for ejaculates in this experiment.

The overall mean %YCBS for all ejaculates was 49.7%. Significant sources of variation in the model were collection frequency ($P<0.0001$) and collection day nested within collection frequency by bull by ejaculate ($P<0.05$) . Table 3.3 contains the

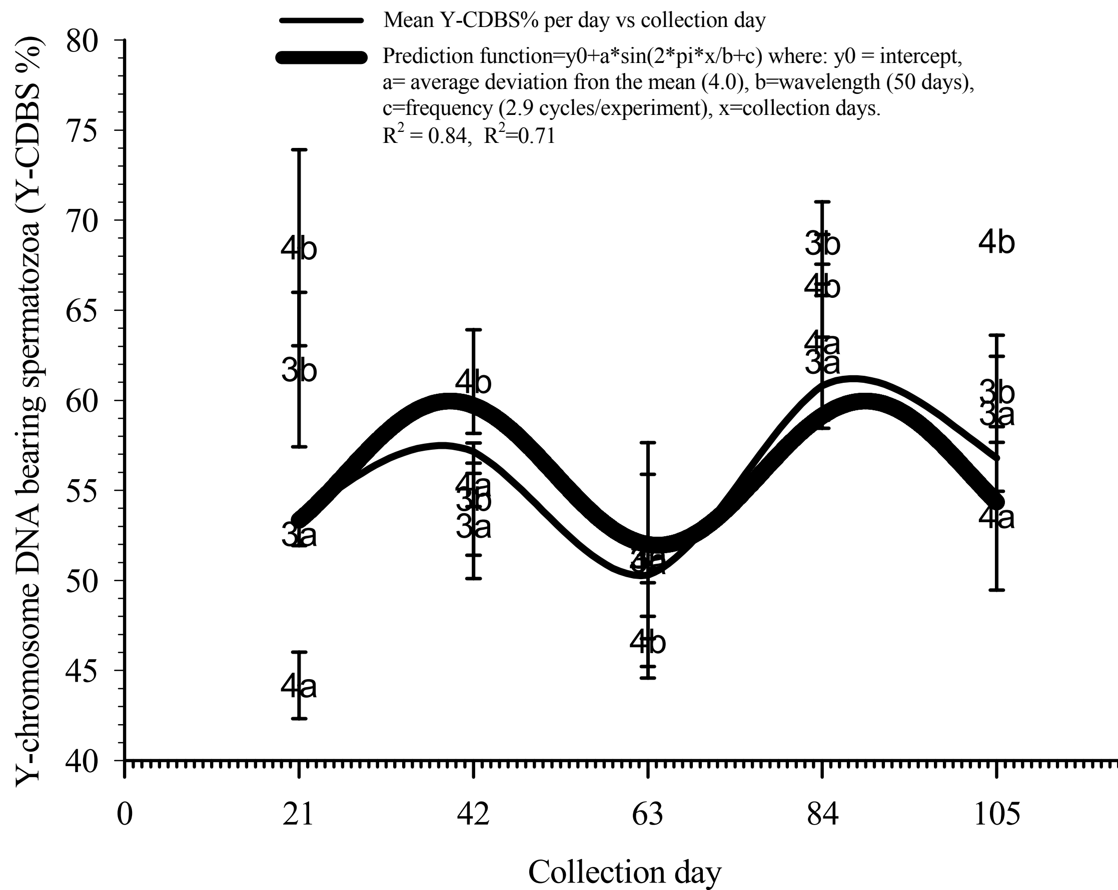


Figure 3.4. Percent Y-chromosome bearing spermatozoa as affected by 21-day collection frequency. Symbols represent mean %Y-CBS for ejaculates (a, b) from each bull (3, 4) on each collection day.

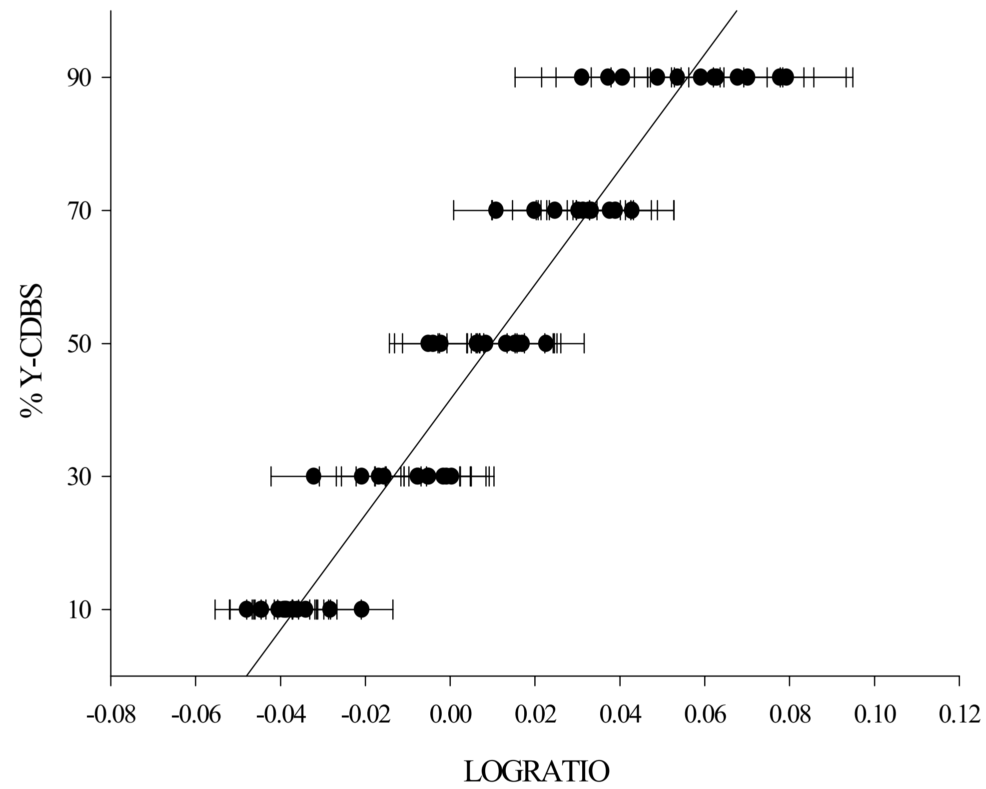


Figure 3.5. Standard curve (R^2 = 93.8%) for prediction of percent Y-chromosome DNA bearing spermatozoa (%Y-CDBS) in ejaculates collected at 7 and 21 day intervals.

analysis of variance for the applied model and Table 3.4 lists the expected mean squares used for the appropriate test for each source in the model.

Table 3.3. Analysis of variance of %Y-chromosome bearing spermatozoa as affected by collection frequency, bull, ejaculate collection day and plate.

Source	df	Mean Square	F-Value	Pr > F
Model	76	21.46	1.70	0.0015
Collection Frequency(Freq)	1	323.59	18.89	<0.0001
Bull(Freq)	2	30.68	1.79	0.1746
Ejaculate(Freq*Bull)	4	20.36	1.19	0.3237
Collection Day (Freq*Bull*Ejaculate)	68	17.13	1.36	0.0513
Plate	1	11.01	0.87	0.3515
Error	226	12.63		
Corrected Total	302			
R^2 (%)	36.4			

Table 3.4. Type III expected mean squares used for hypothesis testing.

Source	Type III Expected Mean Square
Collection Frequency (Freq)	1.0016 x MS(Collection Day(Freq*Bull*Ejaculate - 0.0016 x MS(Error)
Bull(Freq)	1.0012 x MS(Collection Day(Freq*Bull*Ejaculate - 0.0012 x MS(Error)
Ejaculate(Freq*Bull)	1.0012 x MS(Collection Day(Freq*Bull*Ejaculate - 0.0012 x MS(Error)
Collection Day (Freq* Bull*Ejaculate)	MS(Error)
Plate	MS(Error)

Collection frequency may affect %Y-CBS via an epididymal sedimentation phenomenon. Periods of sexual rest between collections may allow some separation of

spermatozoa based on rate of migration through the epididymus (Johnson, 1994; Chandler et al., 1998). Any differences in migration rate between X- and Y-bearing spermatozoa would be due solely to differences in mass since spermatozoa are immotile in the epididymus. Collection of ejaculates at regular, shorter intervals could result in emptying of the extragonadal reserve before any relatively large degree of separation occurs. Figure 3.6 depicts %Y-CBS least squares means for the 7- and 21-day collection frequencies. The 21-day collection scheme resulted in a significantly ($P<0.05$) lower %Y-CBS (47.95%) than the 7-day regimen (50.29%). This finding is not consistent with partial separation in the reproductive tract due to the greater mass and slower migration of the X-chromosome bearing sperm cell. Another counterpoint to this hypothesis is that a the ratio of X- and Y-bearing cells in a second ejaculate collected the same day would be expected to migrate in the opposite direction or possibly become enriched in X-bearing spermatozoa. This is evidenced in the lack of a significant effect of ejaculate nested within bull and collection regime in this experiment.

Figures 3.7 and 3.8 plot %Y-CBS by collection day. The general downward trend in %Y-CBS over time suggests a possible role of testicular function in the variation in the Y-bearing cells over time. If the ratio of X- and Y-CBS produced in the testicle indeed fluctuates, a correctly designed collection regime could take advantage these changes to obtain ejaculates skewed in either direction.

Data in this experiment suggest a relationship between collection regime and %Y-CBS in bull ejaculates. While collection interval significantly affects %Y-CBS in ejaculates over time, the mechanism by which this occurs is not known. Further research

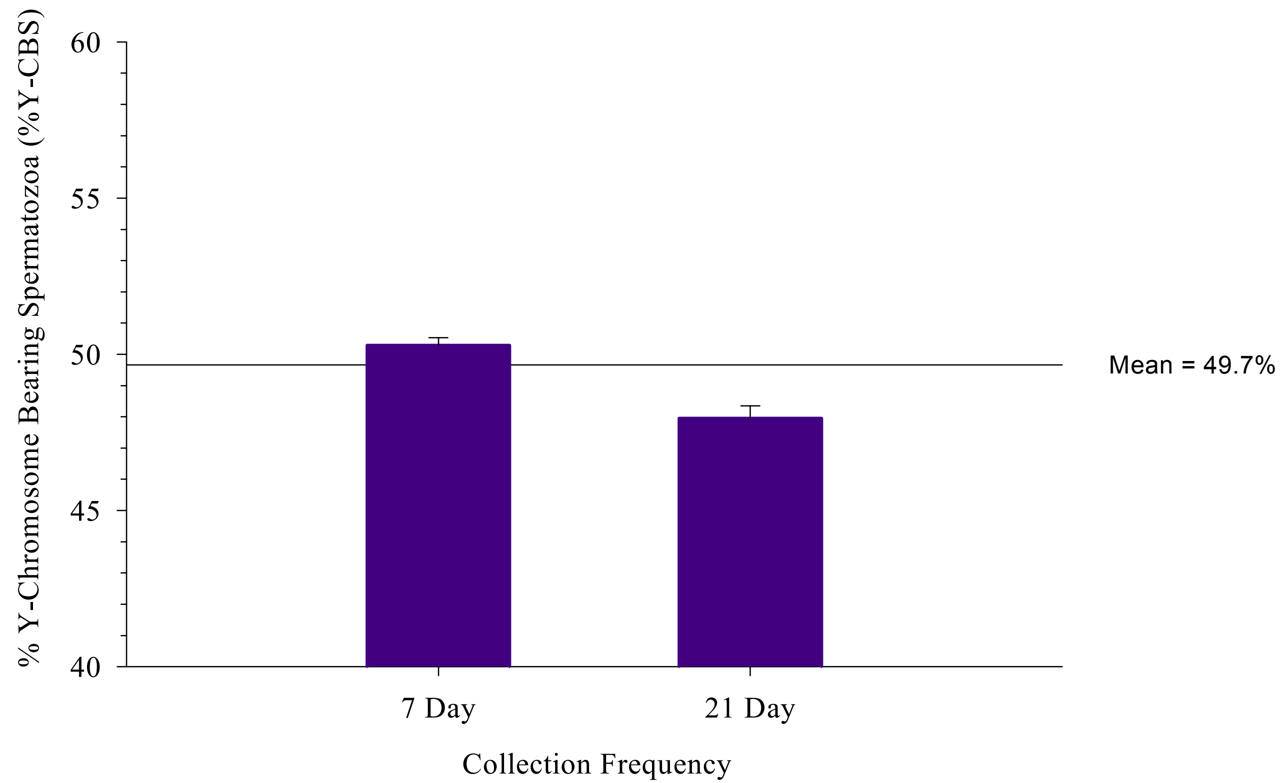


Figure 3.6. Percent Y-chromosome bearing spermatozoa least squares means for 7- and 21-day collection frequencies.

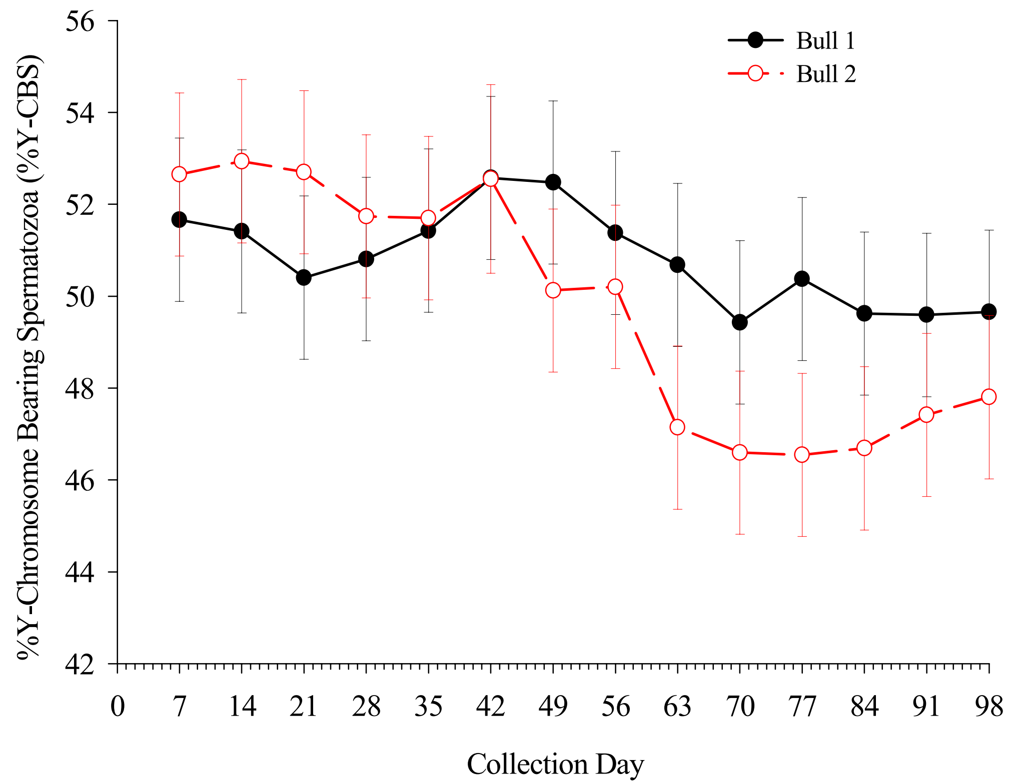


Figure 3.7. Percent Y-chromosome bearing spermatozoa least squares means for 7-day collection frequency.

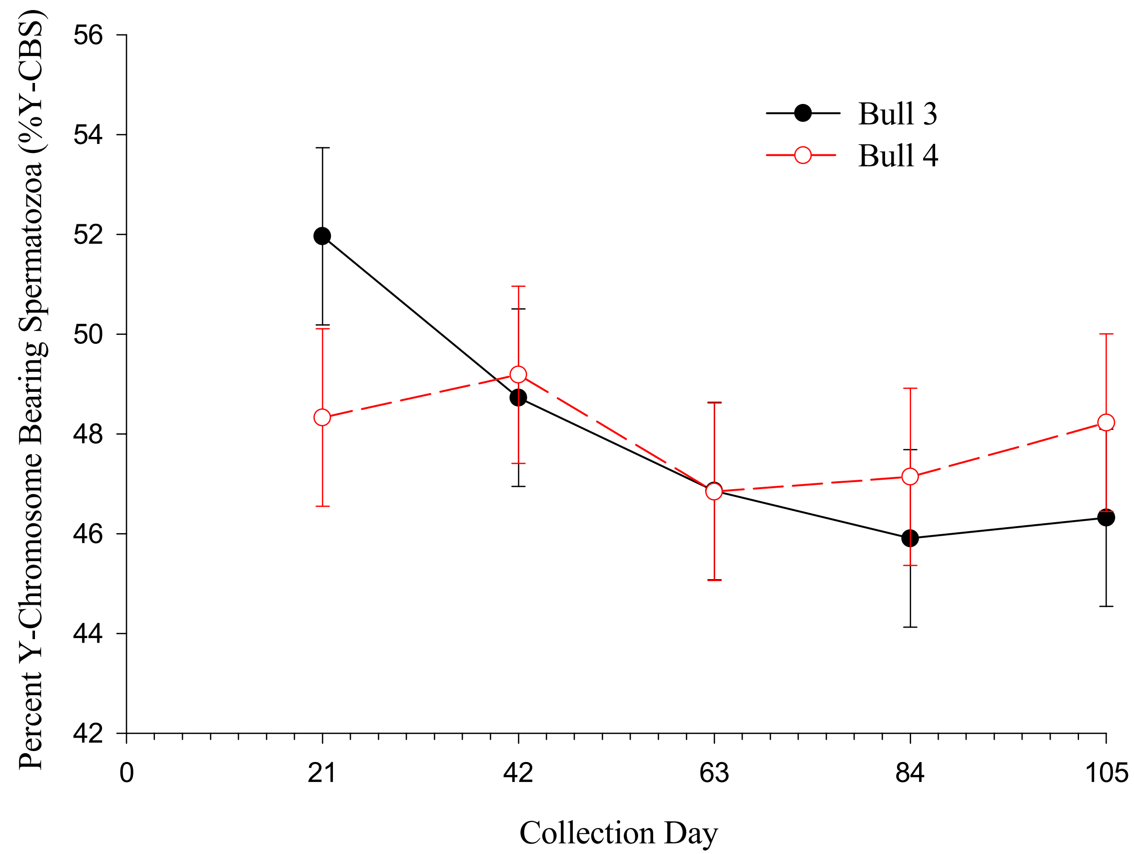


Figure 3.8. Percent Y-chromosome bearing spermatozoa least squares means for 21-day collection frequency.

is warranted to elucidate the basis for differences in the sex chromosome composition of bull ejaculates.

In conclusion, these studies partially elucidate that different collection frequency regimes have a significant contribution to the variation in percent Y-chromosome bearing spermatozoa found in successive ejaculates within individual bulls. There seemed to be some association between sexual rest and a large amount of variability in %Y-CDBS, thus, implying some epididymal function. The conventional PCR experiment illustrated a sinusoid response with a period of about 13 days for bulls collected at a 7-day collection schedule. Those bulls collected at a 21-day interval also responded in a cyclic manner with a period of about 60 days. Perhaps the observed variation resulted from a combination of both heterogeneous testicular function and epididymal function. Additional research is needed to further delineate the mechanism which causes the %Y-CBS cyclicity. Also more work is needed to clarify a management scheme for bulls to maximize variation in %Y-CBS. Ejaculates produced using this undetermined scheme could be selected by PCR screening to manipulate the sex ratio in producers' calf crops.

CHAPTER 4

ASSESSMENT OF SEX CHROMOSOME COMPOSITION OF BOAR EJACULATES USING CONVENTIONAL AND REAL-TIME PCR TECHNIQUES

INTRODUCTION

Natural variation in %Y-CBS in bull ejaculates was established by experiments in Chapter 3. Collection regime influenced %Y-CBS in both experiments, suggesting differential migration of spermatozoa in the epididymus based on head size, heterogenous testicular function or a combination. Ejaculate within bull by collection frequency was a significant ($P<0.07$) source of variation in the conventional PCR experiment. If naturally skewed ejaculates could be selected for the purpose of altering the secondary sex ratio, many segments of animal agriculture would benefit.

Two experiments were designed to evaluate boar ejaculates for %Y-CBS using conventional and real-time PCR techniques. Samples were obtained from boar ejaculates which were subsequently used for artificial insemination of sows in research and commercial breeding programs. After farrowing, litter data was collected and compared to predicted values for %Y-CBS to evaluate the association between the %Y-CBS in ejaculates and the percent male piglets in the resulting offspring.

MATERIALS AND METHODS

Semen Collection

Five boars from a university research farm and 12 boars from a commercial swine farm were collected over a 6 month period via the gloved hand technique. All boars were in rotational breeding use prior to initiation of the study. Semen samples for analysis

were collected by subsampling 67 extended porcine ejaculates before use for artificial insemination.

DNA Extraction

A volume of extended semen containing approximately 15 million spermatozoa from each ejaculate was washed, counted by hemacytometer and aliquots from each ejaculate were combined to construct a pooled sample containing equal numbers of sperm from every ejaculate in the study. Each ejaculate and the pooled sample was extracted by a modified protocol of Aravindakshan et al. (Aravindakshan et al., 1998). Samples were washed twice with 2.9% sodium citrate solution and resuspended in 50EC lysis solution (Aravindakshan et al., 1998) and incubated at 50EC for 30 minutes. Proteinase K (Qiagen Inc., Valencia, CA) (5Fl) was added to the solution and incubated at 50EC for at least 2 hours or overnight. One volume of phenol:chloroform was added and the tubes gently shaken every 3 minutes for 15 minutes at room temperature. The samples were then centrifuged at 15,500 X g for 3 minutes, the organic phase aspirated, and the phenol:chloroform step repeated. Two volumes of ice cold absolute ethanol were added to the remaining aqueous phase and shaken to precipitate the DNA. Samples were centrifuged for 1 minute at 15,500 X g and the ethanol carefully decanted. DNA pellets were dried in a vacuum until clear and resuspended in sterile water by incubating at 37EC with gentle agitation until dissolved. Nucleic acid concentration was determined by spectrophotometry at 260/280 nm.

Conventional PCR

Primers were designed for amplification of two sequences in a duplex PCR reaction. One primer set targeted a 151 bp segment of intron of the sex-determining

region Y (SRY) gene (Pomp et al., 1995; Kadandale et al., 2000; Kakinoki et al., 2002) and the second primers flanked a 122 bp segment of intron of the clotting factor IX (F9) gene (Signer et al., 1996). Target sequences were amplified in duplicate tubes using Ready-To-Go beads (Pharmacia-Biotech, Piscataway, NJ), per manufacturer recommendation, from 300 ng spermatozoal DNA. A thermocylcer was programmed as follows: hot start at 95EC, 1 cycle (2 min @ 95EC, 1 min @ 51EC, 2 min @ 72EC), 29 cycles (1 min @ 95EC, 1 min @ 51EC, 1 min @ 72EC), 10 min @ 72EC.

A standard curve was constructed by amplifying each target sequence separately, separating them from genomic DNA and PCR reaction components via gel electrophoresis and purifying the product bands using spin columns . Each product was quantified by spectrophotometry and serially diluted until a dilution was identified that, after amplification, resulted in a band of equal intensity to that produced by 300 ng pooled DNA under identical PCR conditions. This amount of product was equated to the number of copies of both target sequences present in 300 ng of spermatozoal DNA from a sample with a 1:1 X:Y ratio of sperm. Diluted X and Y products were mixed in 4:1, 3:2, 2:3 and 1:4 ratios to yield mixtures representing the 80:20, 60:40, 40:60 and 20:80 points, respectively, on a standard curve. These mixtures were amplified along with unknown samples in each PCR run and electrophoresed on the same gels. A lambda DNA control reaction supplied by the bead manufacturer and a blank reaction containing no DNA were included in each run as a controls for PCR failure and contamination of reaction components.

Electrophoresis

Amplified samples were electrophoresed on 4% agarose gels in 1X TAE buffer. One microliter of EtBr was mixed into the molten agarose before pouring for fluorescent

staining of PCR products. Two 15 tooth combs were inserted into the molten agarose, dividing the gel into upper and lower sections. Two 11.25 Fl aliquots of PCR product from each tube were combined with 5 Fl loading buffer each. Each aliquot was placed in a well and electrophoresed for 1.5 hours at 127 VDC. Samples were placed on the gel in a crossing manner to eliminate any tube by gel position interactions. The upper section pattern was: lane 1, empty; lane 2, 20:80 standard; lane 3-6, unknown ejaculate samples; lane 7, lambda control; lane 8, DNA size marker; lane 9, blank; lane 10-13, unknown ejaculate samples; lane 14, 40:60 standard; lane 15, empty. The order was reversed on the lower gel section with the 60:40 and 80:20 standards in lanes 2 and 14, respectively.

Image Analysis

Images of EtBr stained gel were captured by a previously described method (Chandler et al., 1998). Image analysis was performed using UTHSCSA ImageTool (University of Texas Health Science Center at San Antonio, San Antonio, TX). Captured images were background subtracted before analysis. The boundaries of each band were identified by the software on each image using the same grey level threshold and were assigned object numbers. Integrated density (INGDEN), which is defined as the product of the average grey level and the number of pixels, was calculated by the software for each band.

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Oligonucleotide primer pairs and fluorogenic probes were designed for the SRY and F9 genes using Primer Express software (Applied Biosystems Inc., Foster City, CA). To facilitate comparison of techniques, target sequences for the real-time assay were

designed to overlap as much as possible with those in tha conventional PCR assay. Due to the design constraints imposed by the software, in order to produce primer/probe combinations for a standard thermocycler program, target sequence homology between assays was not 100% but was maintained above 70% for both targets.

Duplex reaction tubes for unknowns contained 300 ng spermatozoal DNA, 25 Fl PCR Master Mix (Applied Biosystems Inc., Foster City, CA), 900 nM each primer and 300 nM probe, q.s. sterile water to a total volume of 50 Fl. All reactions were amplified in duplicate tubes.

A standard curve was constructed by amplifying each target sequence separately, separating them from genomic DNA and PCR reaction components via gel electrophoresis and purifying the product bands using spin columns. Each product was quantified by spectrophotometry and serially diluted until a dilution was identified that, after amplification, resulted in a C_T approximately centered among C_T's produced by 300 ng pooled DNA under identical PCR conditions. This amount of product was equated to the number of copies of both target sequences present in 300 ng of spermatozoal DNA from a sample with a 1:1 X:Y ratio of sperm. Diluted X and Y products were mixed in 4:1, 3:2, 2:3 and 1:4 ratios to yield mixtures representing the 80:20, 60:40, 40:60 and 20:80 points, respectively, on a standard curve. These mixtures were amplified in triplicate along with unknown samples on each plate.

Litter Data Collection

Litter data was collected on 94 litters resulting from 28 ejaculate samples and assayed in this experiment. Percent male piglets was calculated on an ejaculate basis for comparison to %Y-CBS results.

Statistical Analysis

In the conventional PCR study, log of the ratio of X INGDEN versus Y INGDEN was calculated for each standard curve point estimate. An inverse regression analysis (Ryan, 1997) of the 36 curve point estimates was used to obtain prediction equations for calculating sex ratio in each ejaculate. Predicted values for ejaculates were analyzed using the Mixed Model option of the General Linear Model procedure in the SAS statistical package, Version 8 (SAS User's Guide: Statistics, 1999). Ejaculate source of variation was tested with an adjusted error term. Other effects in the model were tested with random error. Least squares means are presented where significance occurred. The model included the following:

$$Y_{ijk} = \mathrm{F} + \mathrm{Boar}_i + \mathrm{Ejaculate(Boar)}_{i(j)} + \mathrm{Tube(Ejaculate)}_{j(k)} + \text{Sum of Integrated Densities}_{ijk} + e_{ijk}$$

where:

Y_{ijk}	=	predicted percent Y-chromosome bearing spermatozoa
μ	=	overall mean
Boar_i	=	fixed effect of boar i
$\mathrm{Ejaculate(Boar)}_{i(j)}$	=	random effect of the jth ejaculate from the ith boar
$\mathrm{Tube(Ejaculate)}_{j(k)}$	=	random effect of the kth PCR tube of the jth ejaculate
Sum of Integrated $\mathrm{Densities}_{ijk}$	=	linear covariate to account for trends in total fluorescent intensity
e_{ijk}	=	random error

In the real-time experiment, the logs of the ratios of X C_T versus Y C_T was calculated for each standard curve point estimate. Inverse regression of standard curve

points and analysis of predicted values were performed as in the conventional PCR assay. Ejaculate source of variation was tested with an adjusted error term. Other effects in the model were tested with random error. Least squares means are presented where significance occurred. The model included the following:

$$Y_{ij} = F + Boar_i + Ejaculate(Boar)_{i(j)} + e_{ij}$$

where:

Y_{ij} = predicted percent Y-chromosome bearing spermatozoa

F = overall mean

$Boar_i$ = fixed effect of boar i

$Ejaculate(Boar)_{i(j)}$ = random effect of the jth ejaculate from the ith boar

e_{ij} = random error

Pearson product moment correlation analysis was performed on %Y-CBS and percent male piglets using the CORR procedure in the SAS statistical package.

RESULTS AND DISCUSSION

Conventional PCR

The overall mean %YCBS for all ejaculates was 50.26%. Significant sources of variation ($p<0.05$) in the model were boar, ejaculate nested within boar and sum of integrated densities. Table 4.1 includes the analysis of variance for %YCBS. Table 4.2 lists the expected mean squares used the appropriate test for each source on the model. The standard curve used for prediction of %Y-CBS in individual boar ejaculates is illustrated in Figure 4.1.

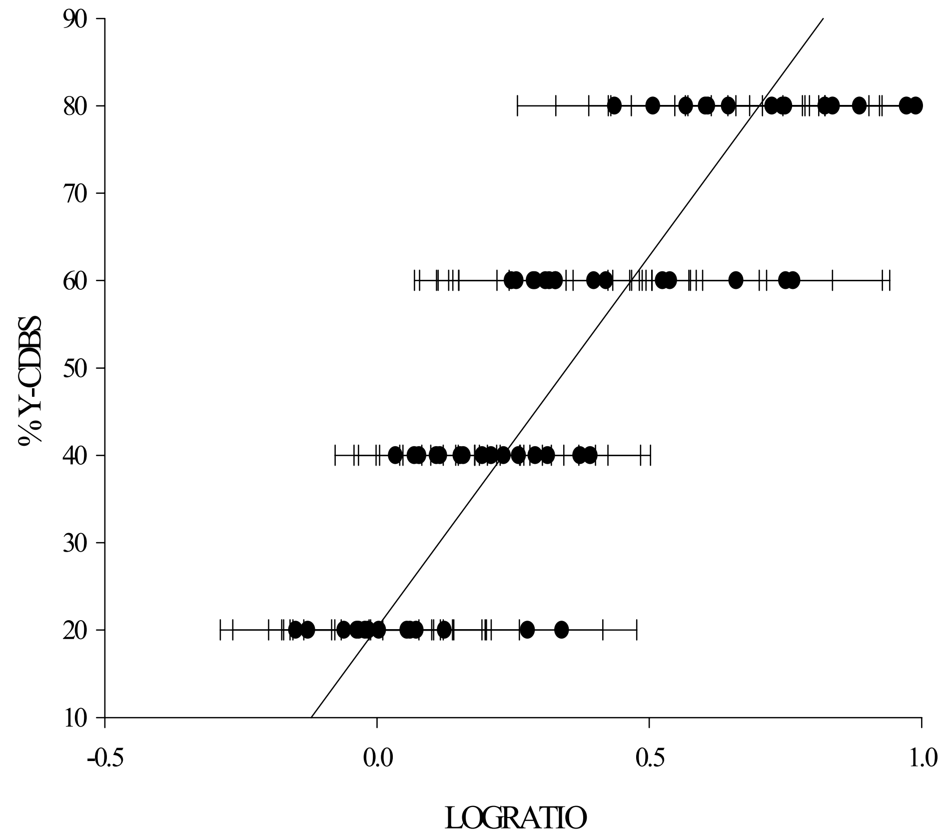

Figure 4.1. Standard curve ($R^2 = 74.4\%$) for prediction of percent Y-chromosome DNA bearing spermatozoa (%Y-CDBS) in ejaculates evaluated by conventional PCR techniques.

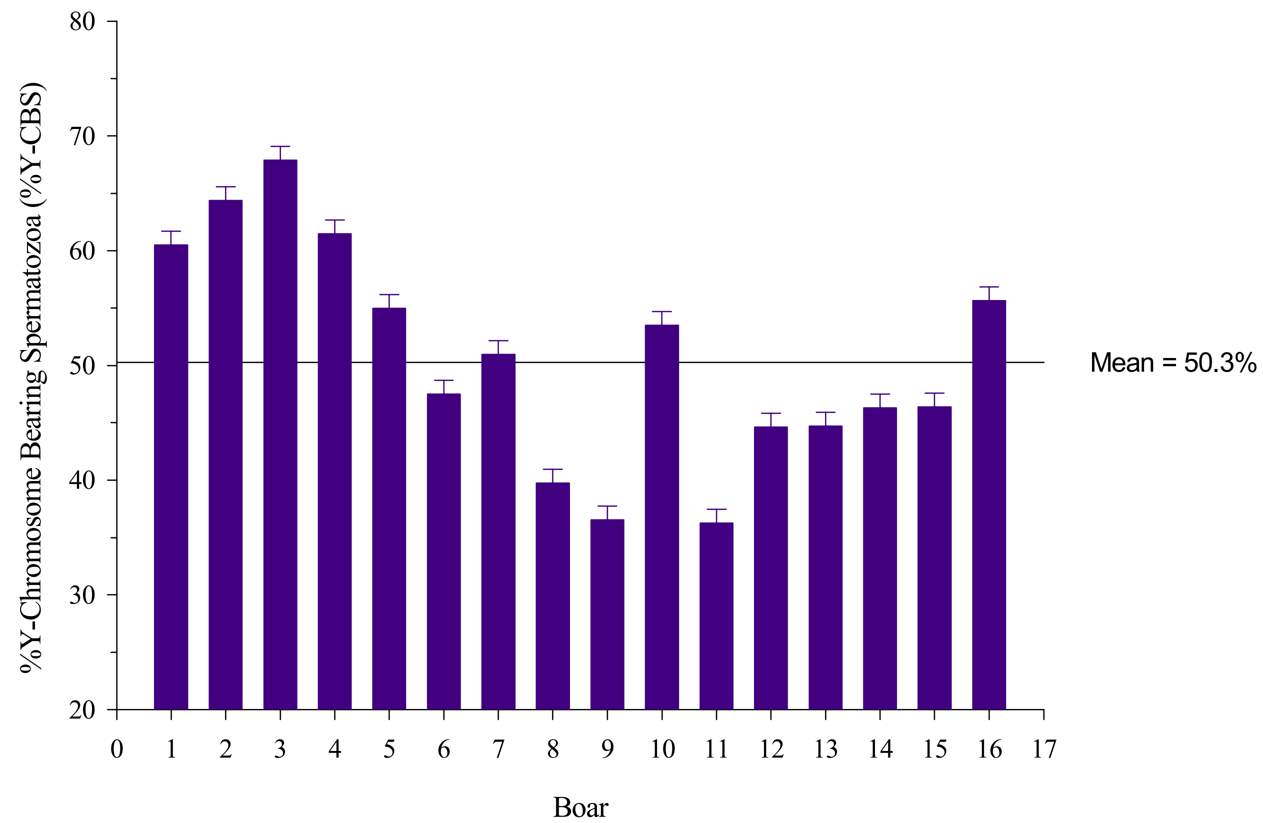

Figure 4.2. Percent Y-CBS least squares means for boars evaluated by conventional PCR techniques.

Figure 4.2 depicts %Y-CBS least squares means for each boar studied. The significant (P<0.002) contribution of boar to the total variance in the model may be due to

Table 4.1. Analysis of variance of %Y-chromosome bearing spermatozoa as affected by boar, ejaculate and PCR tube.

Source	df	Mean Square	F Value	Pr > F
Model	77	570.08	10.38	<0.0001
Boar	15	1078.76	3.04	<0.0022
Ejaculate(Boar)	42	366.56	6.67	<0.0001
Tube(Ejaculate)	10	44.55	0.81	0.6183
Sum of Integrated Densities	1	1494.12	27.2	<0.0001
Residual	189	54.92		
Corrected Total	266			
R^2 (%)	80.9			

Table 4.2. Type III expected mean squares used for hypothesis testing.

Source	Type III Mean Square
Boar	0.9631 x MS(Ejaculate(Boar)) + 0.0369 x MS(Error)
Ejaculate(Boar)	MS(Error)
Tube(Ejaculate)	MS(Error)
Sum of Integrated Densities	MS(Error)

the random (with respect to time) sampling method employed. Boars in this study were active in commercial breeding programs at commencement of the sampling period. If proposed physiological mechanisms for fluctuations in sex chromosome composition of ejaculates (Chandler et al., 1998) are valid, detection of patterns indicative of such would be difficult in the essentially random time points at which boars in this experiment were collected. Boars used more heavily in the breeding program would be subjected to shorter

collection intervals, thus obliterating skews in %Y-CBS due to differential epididymal migration. Less active boars would be collected at greater intervals and would be expected to produce a greater number of skewed ejaculates. The lack of a regular collection regime in this study would also mask any discernable pattern of testicular variation in the production of Y-bearing spermatozoa. Figure 4.3 graphs the %Y-CBS least squares means for ejaculates nested within boars. Sampling method may also be responsible for the significant (P<0.0001) portion of total variance attributable to the ejaculate nested within boar term.

The main goal of this experiment was to evaluate the association of predicted %Y-CBS values from a duplex conventional PCR assay combined with image analysis with the secondary sex ratio of swine litters resulting from assayed ejaculates. Correlation analysis of litter data derived from 28 ejaculates in this study resulted in a weak (Corr = 0.12), nonsignificant (P= 0.54) positive relationship between predicted %Y-CBS and percent male piglets. Figure 4.4 depicts this relationship.

Possible explanations for this lack of association could include error introduced by post-amplification processing. There is a considerable amount of sample manipulation required in the electrophoretic and imaging processes. It is possible that sources of variation not accounted for in the model exist and affect the predicted values for %Y-CBS. However, this seems unlikely since many precautions were taken to minimize the effect of known sources of variation related to image analysis of gels (Dewey et al., 1994; Bell et al., 1998) and the inclusion of appropriate terms in the statistical model.

Factors unrelated to the experimental design potentially affect the association between %Y-CBS and percent male on litters. Maternal effects on the secondary sex ratio may account for a portion of the discrepancy. In sows, many more embryos are conceived

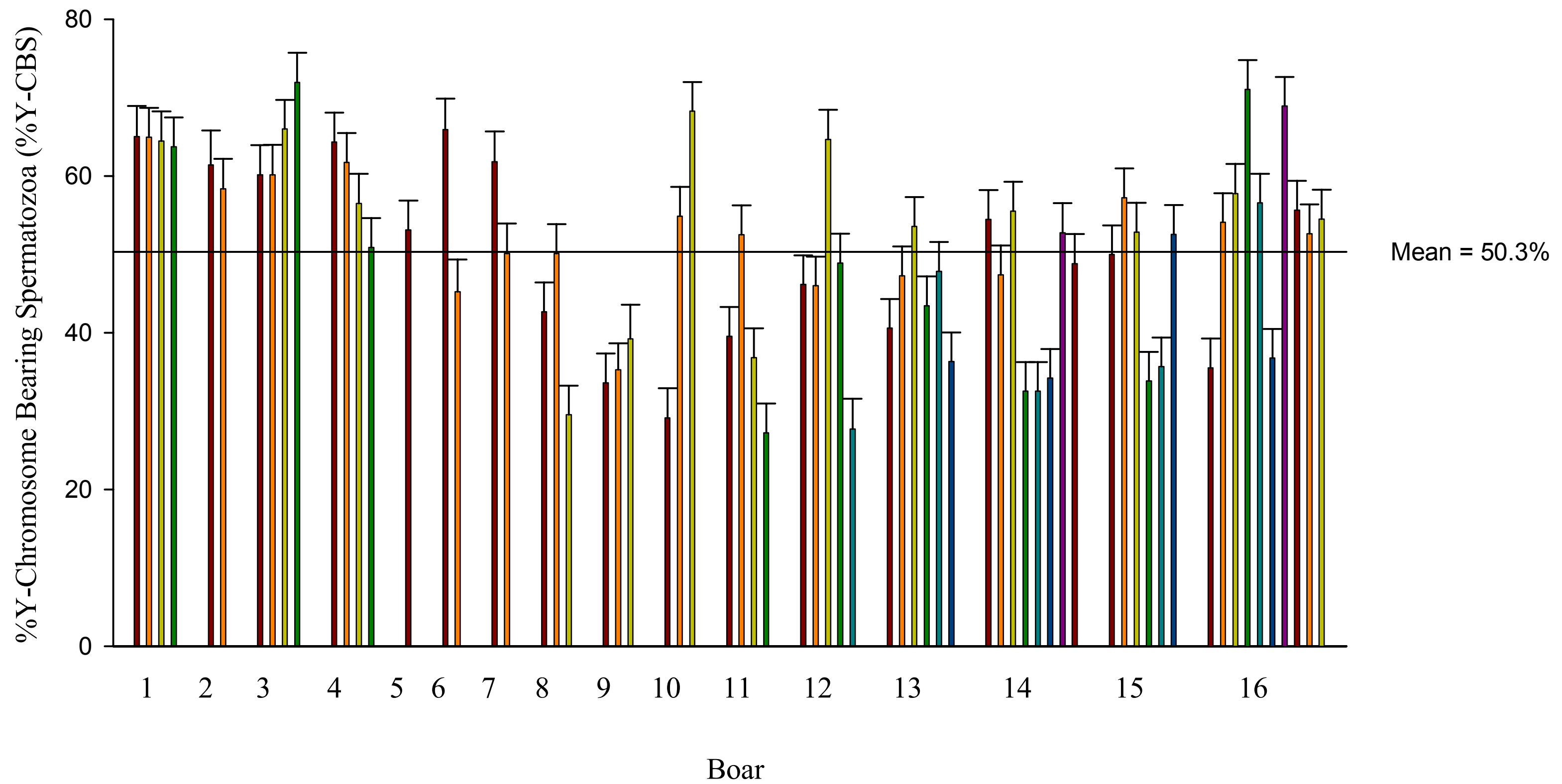

Figure 4.3. Percent Y-CBS for ejaculates (grouped by boar) evaluated by conventional PCR teschiques.

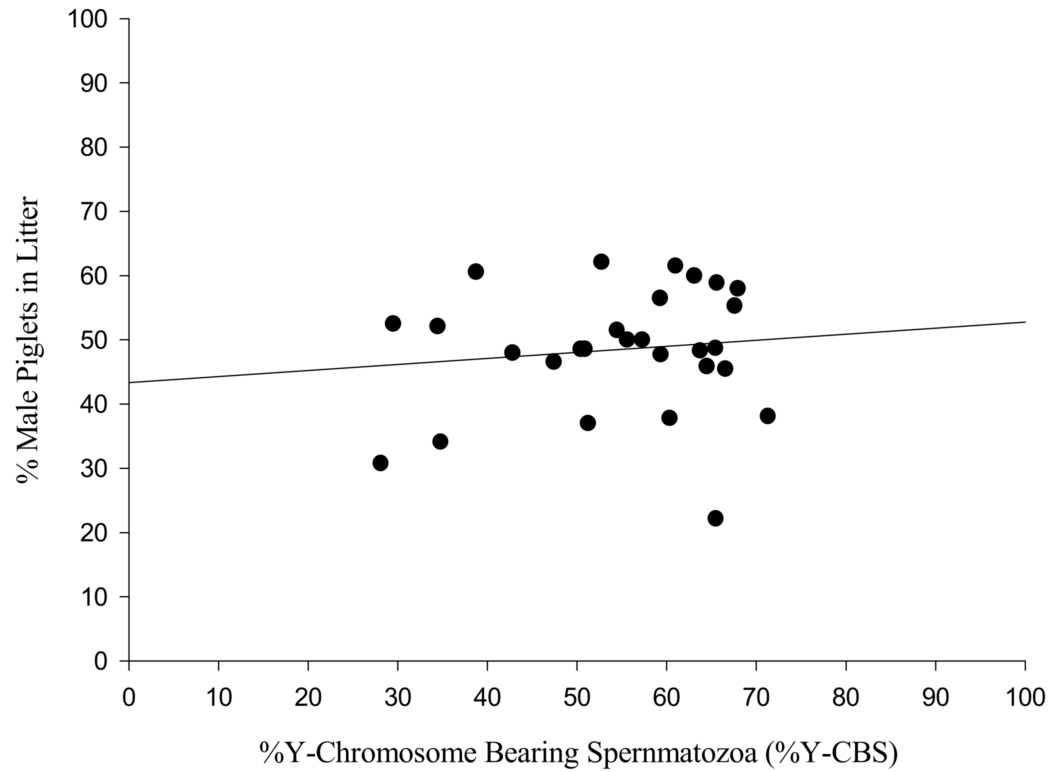

Figure 4.4. Percent Y-CBS vs. percent male piglets in litters resulting from conventionally assayed ejaculates.

than piglets born (Perry and Rowlands, 1962; Pope and First, 1985; van der Lend and Soede, 1994; Roberts et al., 1996). Gender differences in the rate of blastocyst development, with males advancing faster, may render one sex more vulnerable to death when faced with a limited time window for successful implantation (Krackow, 1995a, 1995b, 1997). There is also evidence for sex-specific fetal mortality resulting from limited uterine space later in pregnancy (Chen and Dziuk, 1993). It has also been suggested that high levels of estrogen believed to occur in dominant sows may lead to elective death of slower developing fetuses around day 11-12 of gestation (Roberts et al., 1996).

Timing of insemination has been speculated to modify the expected secondary sex ratio. Evidence exists that varying the timeing of insemination relative to ovulation could skew the population of spermatozoa reaching the site of fertilization toward one gender (Rorie et al., 1999).

Real-time PCR

The overall mean %YCBS for all ejaculates was 50.3%. The significant source of variation ($p<0.0001$) in the model was ejaculate nested within boar. Table 4.3 includes the analysis of variance for %Y-CBS. Table 4.4 lists the expected mean squares used the appropriate test for each source in the model. The standard curve used for prediction of %Y-CBS in individual boar ejaculates is illustrated in Figure 4.5.

Figure 4.6 graphs the least squares means, grouped by boar, for ejaculates assayed. The coefficient of variation for the model in this experiment (7.2%) was reduced when compared to the coefficient in the conventional PCR study. Explanations for increased

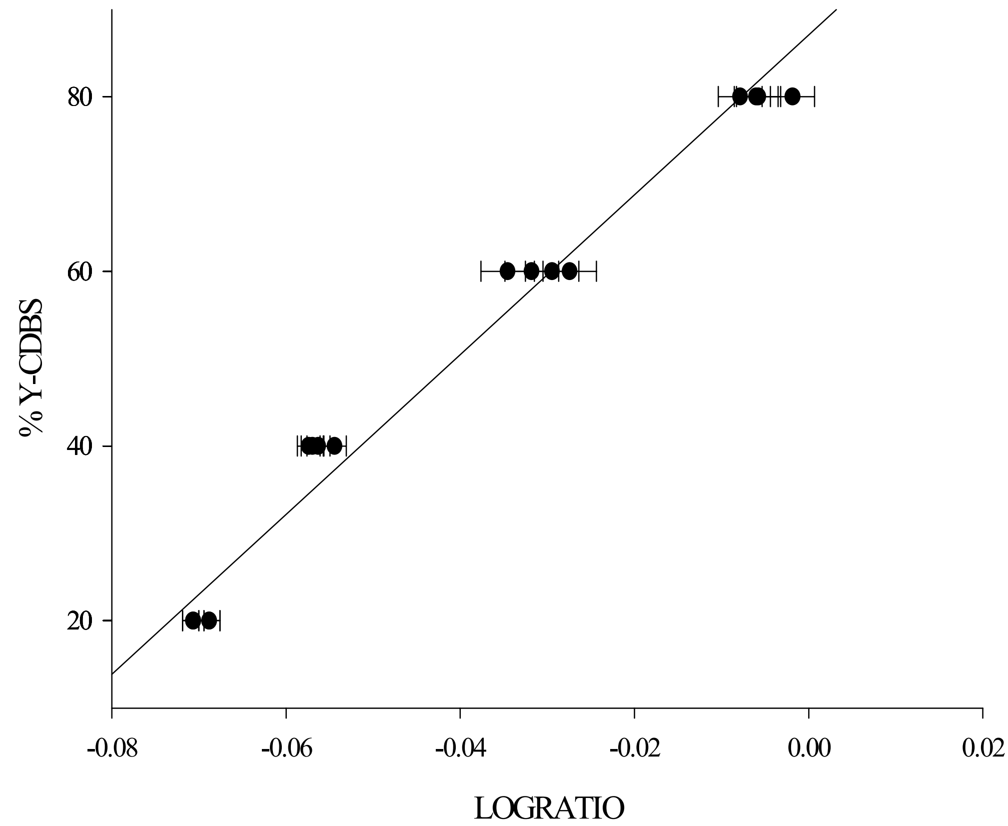

Figure 4.5. Standard curve ($R^2 = 97.6\%$) for prediction of percent Y-chromosome DNA bearing spermatozoa (%Y-CDBS) in ejaculates evaluated by real-time PCR techniques.

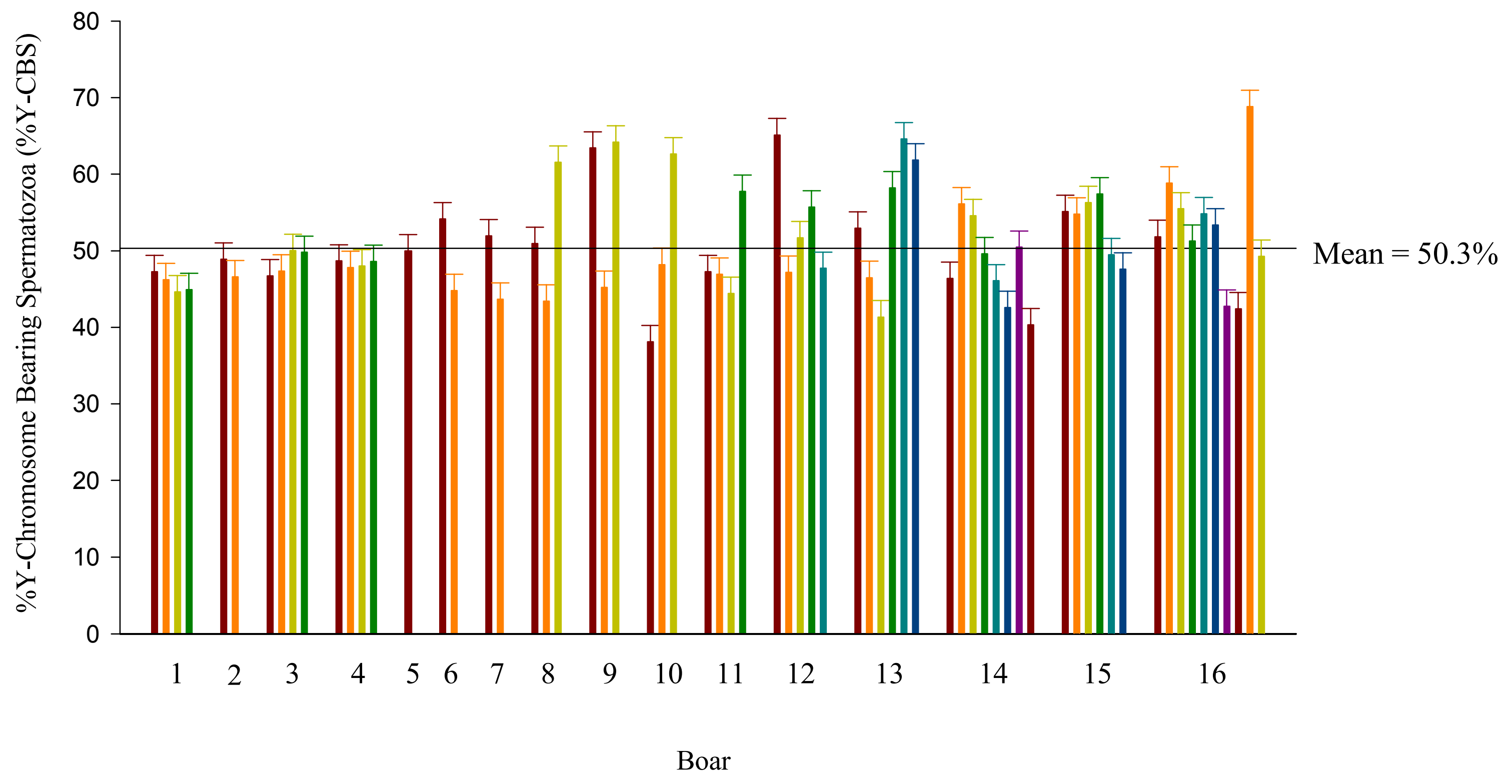

Figure 4.6. Percent Y-CBS for ejaculates (grouped by boar) evaluated by real-time PCR techniques.

precision may include the elimination of the error introduced by post amplification processing of replicate observations and the increased specificity of the fluorescent reporter in realtime reactions.

Table 4.3. Analysis of variance of %Y-chromosome bearing spermatozoa as affected by boar and ejaculate.

Source	df	Mean Square	F Value	Pr>F
Model	66	100.86	9.81	<0.0001
Boar	15	89.17	0.85	<0.6151
Ejaculate(Boar)	51	104.30	10.14	<0.0001
Error	67	10.29		
Corrected Total	133			
R2 (%)	90.6			

Table 4.4. Type III expected mean squares used for hypothesis testing.

Source	Type III Mean Square
Boar	MS(Ejaculate(Boar))
Ejaculate(Boar)	MS(Error)

Increased specificity for the target sequence in realtime PCR assays contributes to greater precision in two ways. Virtually all PCR reactions exhibit some degree of nonspecific amplifcation. Mispriming at loci other than the intended target and/or formation of primer dimers add to the total amount of nucleic acid present in the reaction tube over the course of an amplification. The mixture in the reaction tube also includes the original templet DNA, usually genomic, and the large partial copies of this templet produced by the linear amplification phase of the reaction. In conventional PCR techniques employing intercalating or minor groove binding fluorescent dyes, all double-

stranded nucleic acids present are labeled and contribute to the reporting signal. Depending on the starting amount of genomic DNA and the severity of nonspecific amplification, this could introduce appreciable amounts of error into assays.

This disadvantage is circumvented in real-time PCR assays by annealing a fluorgenic probe between the primer binding sites on the sequence of interest. This probe only contributes to the reporting signal when cleaved by polymerase. Thus, signal from sources other than specific amplification of the intended target is eliminated.

Increased measurement precision may partially explain better association of the real-time assay with the secondary sex ratio of swine litters resulting from assayed ejaculates. Correlation analysis of litter data derived from 28 ejaculates in this study resulted in a stronger (Corr = 0.52), and significant (P= 0.004) positive relationship between predicted %Y-CBS in ejaculates and percent male piglets in the corresponding litters. Figure 4.7 depicts a scatterplot of predicted %Y-CBS and percent male piglets for the real-time experiment.

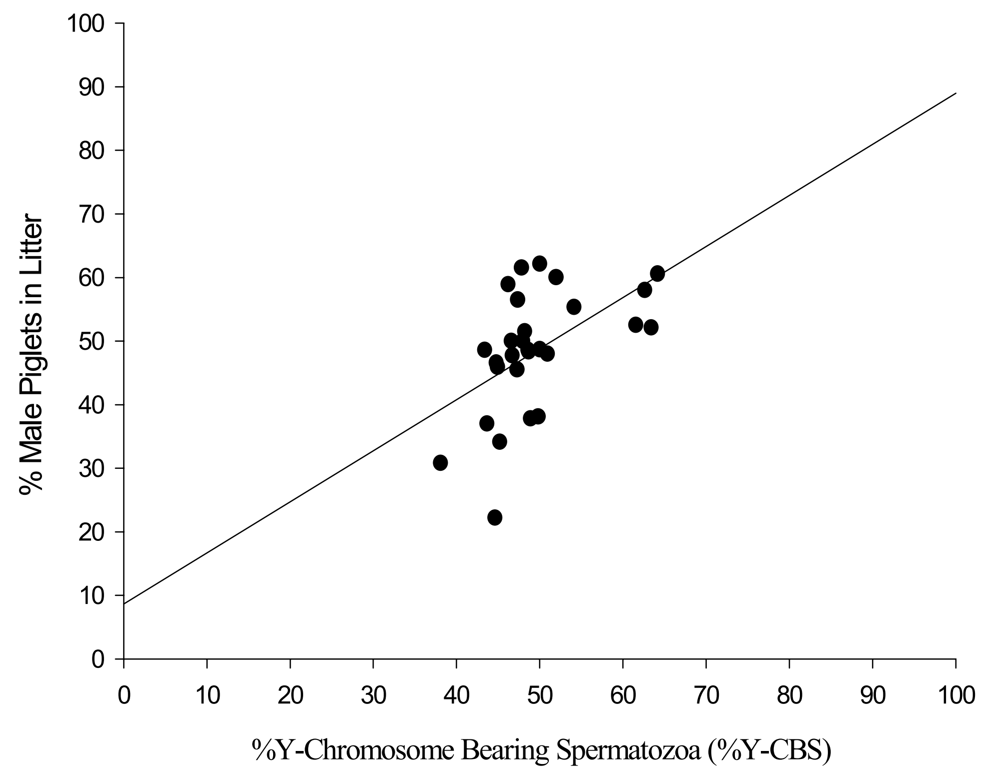

Figure 4.7. Percent Y-CBS vs. percent male piglets in litters resulting from real-time assayed ejaculates.

CHAPTER 5

CONCLUSIONS

Variation in %Y-CBS across ejaculates within bull and boar has been shown to exist (Chandler et al., 1998). Two PCR assays were employed to quantify the naturally occurring variation present in random boar ejaculates. The same techniques were used to assess the effect of two semen collection regimes on %Y-CBS over time in bulls. Mean % Y-CBS of individual boar ejaculates evaluated by conventional PCR analysis ranged from 27% to 72%. Real-time analysis of the same ejaculates resulted in a range of 38% to 64%. Ranges for bull ejaculates were 18-68% and 46-53% for the gel and real-time assays, respectively. Depending on the viewpoint chosen, the degree of dispersion in estimates of %Y-CBS stems from one of several causes.

When comparing gel assays, the wider variation in bull ejaculates could be attributed to a testicular and/or epididymal function. The regular fluctuations noted in bull ejaculate means and the apparent coincidence with spermatogenic cycle dynamics is a compelling argument for a physiological, testicular basis for variation in %Y-CBS. The significant effect of collection frequency supports this assumption. Perhaps optimization of collection timing could result in maximal variation in %Y-CBS in individual ejaculates. Identifying heavily skewed specimens and using them to alter the secondary sex ratio would have great benefit for animal agriculture, as well as human medicine.

Considering the magnitude of variation in estimates of %Y-CBS produced from real-time PCR assays of bull and boar ejaculates suggests a different cause for the discrepancy. The tighter range of estimates obtained in the bull samples argues that the

regularity of the collection regime served to reduce the variation in the proportion of Y-bearing cell in the ejaculates evaluated. Regularly emptying the extragonadal reserve may diminish the effect of any epididymal settling that would occur over longer periods of sexual rest. Boars in these experiments were collected on a somewhat random time basis during the course of a breeding season. Perhaps the greater variation observed in boar ejaculates is a result of differing lengths of time between collections, allowing a physiological phenomenon, such as, epididymal settling to cause random fluctuation in %Y-CBS . The significant effect of boar in the real-time analysis lends credibility to this supposition. In addition, there was not a significant bull effect in the real-time analysis, however bull was nested within collection frequency with only two bulls per treatment. Larger sample size could give a different outcome.

Comparison of conventional and real-time PCR techniques performed on the same samples revealed greater precision in the real-time assays. The coefficients of variation for real-time assays were approximately half of those for the conventional PCR technique. The range of estimates for %Y-CBS was much narrower in the real-time assay for both bulls and boars. The coefficients of determination for the standard curves generated in these experiments illustrates the increased precision possible with real-time PCR. In the boar assays the R^2 increased from 74.4 to 97.6% for the conventional and real-time assays, respectively. The coefficient also improved in the bull assays from 85% for the conventional standard curve to 93.8% for the real-time method.

The coefficients of determination for the models used to analyze treatment effects on predicted %Y-CBS were also much higher for the real-time bull and boar analyses with fewer terms included in the model. This suggests that variation exists in the

conventional techniques that was not accounted for, even with more complex statistical models.

Correlation analysis yielded nonsignificant ($P < 0.55$) association (Corr = 0.12) between estimates of %Y-CBS in boar ejaculates and percent male piglets in litter resulting from subsequent breedings in the conventional PCR experiment. Association for real-time estimates was greater (Corr = 0.52) and was significant ($P < 0.004$). Many factors may contribute to the lack of strong association in both assays including maternal modulation of sex ratio (James, 1989; Drickamer, 1990; Clark et al., 1993; Clark and Galef, 1995; James, 1998).

Further research is warranted to elucidate the true nature of variation in the primary sex ratio. Greater numbers of animals collected on a custom regime aimed at maximizing variation in %Y-CBS may give clues to the physiological basis for the fluctuations noted in these studies. Closer examination of the spermatogenic cycle from a "whole testicle" viewpoint could give insight to the variation at the cellular level. Research designed to define epididymal dynamics as they relate to sperm X- and Y-bearing sperm movement possibly holds some answers. The current studies show that appreciable variation in %Y-CBS exists. How to best take advantage of it is yet to be determined.

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Mr. John Chandler on behalf of JB Paul

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VITA

Julius Braden Paul was born to Freddie Wilson Paul and Tresa Lynn Latham Paul in Bossier City, Louisiana, on April 4, 1970. He was raised on a beef/dairy farm near Converse, Louisiana, where he developed an understanding and love for animals and the livestock industry. He graduated as salutatorian from Converse High School, in Converse, Louisiana, in May 1988.

He received a bachelor of science degree in agriculture, with a major in animal science, from Louisiana State University in May, 1992. He entered the graduate school at Louisiana State University and completed 2 years of study and an internship at Trans Ova Genetics, Sioux Center, Iowa. He received a master of science degree in agriculture, with a major in reproductive physiology, from Louisiana State University in May, 2000. In the interim, he was employed by the Louisiana Cooperative Extension Service as an assistant county agent in livestock and youth development. He is currently employed as an andrologist at Woman's Hospital in Baton Rouge, Louisiana.