Evaluation of bovine spermatid sex ratio by fluorescent in situ hybridization

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EVALUATION OF BOVINE SPERMATID
SEX RATIO BY
FLUORESCENT IN SITU HYBRIDIZATION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the degree of
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in

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Dairy, and Poultry Sciences

by
Michael Alan Stout
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ABSTRACT

The objective of this study was to use fluorescent in situ hybridization (FISH) to determine if a skew in the sex ratio was present at the level of the bovine round spermatid. Paraffin embedded bovine testicular tissue obtained from biopsies were used to perform FISH, and the seminiferous tubules were examined. Our findings show that the skew does exist within the round spermatids. Analysis of variance on X- and Y-chromosome bearing round spermatids as affected by sex, slide, picture set, and tubule was performed. Sex showed that the variation in round spermatid sex ratio differed between slides \( (P = 0.0001) \). The interaction of sex by slide were indicated to be non-significant \( (P = 0.0815) \). Sex within tubule nested in slide showed a significant deviation in sex ratio \( (P = 0.0122) \). Picture set showed no significant difference in counts, confirming that the counts were consistent \( (P = 0.2096) \). A closer look at the data also showed a 10 to 30% variation in the number of seminiferous tubules with a significant skew per slide. Most the skew was toward the Y-chromosome, but a significant skew toward the X-chromosome was also seen. This skew toward the X-chromosome showed that the skew could be counteracted or reversed.

Meiotic drives have been shown to exist in other species. Meiotic drives are alleles with the ability to increase their probability of being transmitted to the next generation. Although we cannot prove that a meiotic drive exists in bovine, our findings agree with the meiotic drive principle. These drives push the sex ratio of the population in one direction. Modifiers attached to these drives can counteract or reverse the effect of the meiotic drive. Environmental factors such as crowding, brought on by intense group housing could also have an effect on the expression of these drives. Further investigation
is necessary to completely understand these skews in sex ratio and what factors may affect it.
INTRODUCTION

Attempts to skew sex ratios have probably been around since the dawn of mankind. Aristotle claimed that the sex of the offspring was determined by the heat of the male partner during intercourse. The more heated the passion, the increase in the probability of conceiving a male offspring. He thus told old men to conceive in the summer if they wished to have male heirs (Gilbert, 2003). The ancient Greeks believed that spermatozoa from the left testicle produced girls and spermatozoa from the right testicle produced boys. The myth apparently stood the test of time, since in the 1700s French noblemen would have their left testicle removed to increase the odds of fathering sons. A quote from P.L. Senger’s book Pathways to Pregnancy and Parturition, Senger amusingly proposes that the modern day declaration by males,”I would give my left testicle for a ____” is a sexist comment that devalues the left testicle because it was once thought to produce females only. “Have you ever heard a male say he was willing to give up his right testicle for something?”(Senger, 1999)

Skewed sex ratios have been described in humans by Lobel et al. (1993) and in bull semen per ejaculate by Chandler et al. (1998) using polymerase chain reaction (PCR). The purpose of this study was to determine if the source of the skew originates in the production of semen in the seminiferous tubules. This work was started in the bull by R.P. Amann (1962) when he described testicular germ cell degeneration in the dairy bull. In this study the idea was taken one step further by describing the sex ratio to see if the degeneration could have an effect on the sex ratio of a bull’s offspring. Evaluation of first generation round haploid spermatids before nuclear compaction was performed by fluorescent in situ hybridization (FISH) on paraffin embedded tissue. Fluorescent in situ
hybridization has been used to validate the sorting ability of flow cytometry by Kawasaki et al. (1998) and Rens et al. (2001). Population of X and Y chromosomes are visible in the round spermatids which are direct indicators of X and Y semen ratios produced by that section of seminiferous tubule of that bull at that time. The reason for staining first generation spermatids is that it is the only available time to stain haploid cells before nuclear compaction. Nuclear compaction makes chromosomes unavailable for staining. The reagents used to un-compact spermatids would destroy the morphology of seminiferous tubule tissue. Evaluation of spermatocytes would not provide information on sex ratios since both X and Y chromosomes are contained in each cell.
LITERATURE REVIEW

Overview of Spermatogenesis

As described by Johnson, in his paper *Spermatogenesis in the Bull*, spermatogenesis is the process by which spermatogonia, through a series of divisions, produce spermatozoa (Johnson, 1994). The series of divisions can be broken down into three major divisions: spermatocytogenesis, meiosis, and spermiogenesis.

Spermatocytogenesis is the division of spermatogonia to produce intermediate Type (In) spermatogonia, Type B (B) spermatogonia and preleptotene primary spermatocytes. Meiosis begins with the leptotene primary spermatocytes; they then differentiate through a series of meiotic nuclear configurations known as zygotene (Z), pachytene (P), and then to diplotene (D). The first meiotic division produces secondary spermatocytes, and then the second meiotic division produces Sa spermatids (Sa). Spermiogenesis begins with Sa spermatids, which then differentiate through a series of morphological changes denoted as Sb₁, Sb₂, Sc, Sd₁, Sd₂, and ends in spermiation as spermatozoa. In the bull the three major divisions take 21, 23, and 17 days respectively for a total of 61 days (Johnson, 1994).

During spermatocytogenesis, stem cell spermatogonia will continually produce spermatogonia throughout the mature life span of the male through the process of mitosis. Stem cells also produce committed spermatogonia which will differentiate to produce primary spermatocytes which undergo meiosis. During the preleptotene stage, the primary spermatocytes synthesize DNA for meiosis prophase I, this encompasses the five stages mentioned previously (Johnson, 1994). Meiosis of primary spermatocytes allows the exchange of genetic material between homologous chromosomes and then makes
haploid spermatids. The real beginning of meiotic prophase 1 begins with the leptotene spermatocyte which is distinguished by the crust of chromatin in the nucleus. At the end of this stage, spiralization and contraction of the chromosome has occurred and is finished after these cells contain a tetraploid amount of DNA. Zygotene starts the next stage, when the analogous chromosomes pair off and become thicker while forming into a boutique arrangement (Johnson, 1994). The chromosomes and nuclei become visible when synaptinemal complexes appear between the pairs. During the pachytene stage, which is the longest stage of meiotic prophase, the chromosomes thicken and begin to show longitudinal splits except at the level of centromeres. This is the stage in mammals when crossing over occurs between paired chromosomes. During diplotene stage, duplication of the chromosomes is complete, forming complete tetrads. Finally diakinesis occurs which is extremely rapid, members of the pairs of homologous chromosomes separate into two diploid secondary spermatocytes. Secondary spermatocytes are short lived and divide again to produce cells with a haploid number of chromosomes called spermatids (Johnson, 1994).

Spermiogenesis begins with round spermatids which will become spermatozoa through the elongation of the cell and condensing of nuclear material, which is characterized by the production of the acrosome, flagella and condensed head. SA spermatids reveal the acrosomal vesicle and large Golgi apparatus. SB₁ spermatids form an acrosomal cap when the acrosomal vesicle flattens over the nucleus (Johnson, 1994). SB₂ spermatids are characterized by a distinct tail, elongation of the nucleus, and the appearance of the manchette. SC spermatids have a distinct manchette and a darkened nucleus. SD₁ spermatid nuclei condense further and the manchette disappears, which
indicates SD$_2$ spermatid formation. SD$_2$ spermatids are characterized by the annulus migrating to the distal position and mitochondria move around the mid-piece.

Nuclear condensation is the process by which the chromatin changes from a beaded pattern typical of histones to a smooth fiber due to the exchange of histones with protamines. DNA molecules bind with histones forming nucleosomes. Nucleosomes form beads on DNA which coil and twist to form super coiled chromatin fiber. These fibers further coil forming chromosomes. Protamines are small arginine rich nuclear proteins that take the place of histones in spermatozoa to achieve high chromatin compaction. Further stabilization is achieved with the oxidation of protamine cystine residues to form disulfide bonds (Ward and Coffey, 1991). When spermatids are first formed they have a characteristic round nucleus, which can be stained to reveal the DNA content. This staining reveals that spermatids have one-half the DNA content of secondary spermatocytes or one-quarter of the primary spermatocyte’s DNA content (Setchell, 1978). There is a progressive aggregation of the chromatin granules which produces a highly electron dense nucleus. As this happens the amount of DNA available to staining reduced due to the condensing chromatin. Thus the evaluation of spermatid DNA content should be done at an early stage before nuclear compaction, so the DNA will be readily available for staining (Bizzaro et al., 1998).

In R. P. Amann’s (1962) paper, “Reproductive Capacity of Dairy Bulls”, he describes degeneration patterns in spermatogenesis. His calculations suggest that the theoretical yield of one Type A-spermatogonia is 16 primary spermatocytes and 64 spermatids. Although in his study there was no report of spermatid degeneration, cited in his paper are studies done in the rat by Roosen-Runge (1955) in which they found a 10%
degeneration of young spermatids. Also in this paper, he cites Oakberg’s (1956) study in which he found a 13% loss during the two meiotic divisions (Amann, 1962).

After completion of the meiotic divisions it has been shown that the X and Y chromosome genes within the round and elongating spermatids are transcriptionally active. The majority of genes found on the Y chromosome are transcribed in spermatids. Cytoplasmic bridges allow these gene products to be transported between spermatids thus allowing them to influence one another (Grootegoed et al., 1995).

**Sex Ratio**

Sex ratio is defined as the number of males in a specific population or stage of life per 100 females. Lobel et al. (1993) found a variation in Y-chromosome bearing spermatozoa in humans from 41.9 to 56.7% using polymerase chain reaction (PCR). Chandler et al. (1998) studied the variation in sex ratio between ejaculates by polymerase chain reaction (PCR) in bulls. Ejaculates within bulls had a variation of 24 to 84% ± 9.8 of sperm bearing the Y-chromosome. Ejaculates from the same bull varied in the percentages of male calves from 16 to 72.3%. The results obtained suggest that X and Y bearing spermatozoa are unequally distributed per ejaculate. The use of ejaculates screened by PCR could increase the production of the desired sex of the calf. Alternatively, screening of ejaculates could be used to marginalize the effect of sex ratio skew on an individual producers operational cost.

Collection frequency and their affects on percent Y-chromosome bearing sperm have been studied by Chandler et al. (2002). In their first experiment, six ejaculates from each of five sexually rested bulls were tested by PCR to determine percent Y-chromosome. The first ejaculate ranged from 17%-71% Y-bearing chromosome. The
second ejaculate remained high in percentage but the variation in the third and fourth lessened. This pointed toward an epididymal storage function as the reason for the variation. In their second experiment, bulls were collected at a 7 day or at a 21 day interval (Chandler et al., 2002). There were oscillations in the average percent Y-chromosome bearing spermatozoa across the entire experiment. It was noted in their second experiment that the pattern modulated at a 63 day interval and then repeated. The period or wavelength was established to be approximately 13.5 days. Within a 63 day period, there were 4.2 waves. Afterwards there were 2.4 waves which were equivalent to the pre-63 day period. In spermatogenesis, there are about 4 cycles necessary to complete the formation of spermatozoa. These cycles last around 13.5 days and are known as the length of the spermatogenetic cycle. A periodic overall response was also seen in percent Y-chromosome bearing spermatozoa on a 21 day schedule. The author noted that this is reminiscent of the cyclic nature of spermiogenesis, i.e. the time of the formation of primary spermatocytes to spermiation, reported to be approximately 43 days (Chandler et al., 2002).

Flow cytometry has been used to separate X and Y bearing spermatozoa. This sorting technology is based on total DNA content of the spermatozoa (Johnson, 1999). It has been shown that the sex ratio of spermatozoa after sorting by flow cytometry is of equal proportion (Garner et al., 1983). The limited sorting rate and reduced fertility of sorted spermatozoa has encouraged further research (Johnson and Schulman, 1994).

Secondary sex ratios have been reported in human females. These studies showed that a variety of conditions exist, which can cause a skew in the sex ratio of births. Maternal weight at conception, unfavorable season, environmental pollution, destructive
earthquakes, smoking parents and the age of the mother or father have all been reported to produce a reduction of sex ratio. The mechanisms which cause these skews in sex are still unclear, but seem to indicate that males are more fragile than females, which cause an increase in male embryo abortions in non-optimal conditions (Cagnacci et al., 2004).

Female roe deer, red deer, and other large ruminants in better body condition produce more male than female progeny. Sex ratios of farm raised roe deer on a controlled diet for low and high energy intake were studied. The offspring born to the high energy diet consisted of 75% males, as opposed to 46% male offspring to the low energy diet females (Rosenfeld and Roberts, 2004).

Bacteria have been known to skew the sex ratio of insects. *Wolbachia* and *Spiroplasma* infect their host and cause a variety of effects on their reproductive abilities, such as: male killing, parthenogenesis, feminization, and cytoplasmic incompatibility. *Spiroplasma* infected *Drosophila neocardiini*, ornatifrons, and *paraguayensis* produced all-female broods. This skew in the sex ratio was caused by the cytoplasmically transmitted spiroplasma which killed all the male eggs in the brood. Spiroplasmas are not transmitted in semen, and treatment of infected insects with antibiotics restored the sex ratio back to 1:1 (Montenegro et al., 2006). *Wolbachia* infected *Hypolimnas bolina* (butterfly) also display an extreme sex ratio in favor of the female. The cytoplasmically transmitted male killer skews the female to male sex ratio to 100:1. The death of males causes a 57% reduction in reproductive output of each infected female. The infected females produce both male and female zygotes, but through an unknown mechanism the males die during embryogenesis (Dyson and Hurst, 2004).
The time interval from artificial insemination to ovulation has been reported to skew the sex ratio of calves. Pursley et al. (1998) while using an ovulation synchronization program showed that inseminating cows 24 to 32 hours before ovulation and 0 to 8 hours after ovulation resulted in 61% and 65% more female calves, respectively (Pursley et al., 1998). This data was refuted by Roelofs et al. (2006). In this study they inseminated cows at three intervals including early (between 36 and 20 hours before ovulation), intermediate (between 20 and 8 hours before ovulation), and late (8 hours before and 12 hours after ovulation) with results of 50, 50, and 55%, respectively, on seven day old embryos. The results of this study indicated that the interval between insemination and ovulation had no influence on the sex ratio of seven day old embryos (Roelofs et al., 2006).

**Meiotic Drive**

Meiotic drive as described by Hurst and Pomiankowski (1991) is an allele with the ability to increase its probability of being transmitted to the next generation at meiosis. This meiotic drive is found throughout nature and is described in various species. Meiotic drive elements are often attached to sex chromosomes. If the drive element is attached to a sex chromosome lacking the insensitivity allele it will drive against itself and quickly be eliminated. Sex drive elements will only spread in a population if they are attached to sex chromosomes coupled to insensitivity alleles. A drive on the sex chromosomes will cause an alteration to the offspring sex ratio. Y-linked sex ratio drivers produce male biased offspring, while X-linked drives produce female biased offspring. As the sex linked drive elements spread, the proportion of offspring becomes increasingly biased. Drive elements are more likely to become established in the
active form if attached to sex chromosomes. The primary reason for this is the low frequency or complete absence of recombination between X and Y chromosomes. In sex ratio drive systems there are a number of other loci that enhance or suppress the degree of distortion produced by the drive agent. These loci are called modifiers. Modifiers may be found on all chromosomes (Hurst and Pomiankowski, 1991).

Stalked eyed flies (Cyrtodiopsis dalmanni and C. whitei) demonstrated an extreme female sex ratio due to a meiotic drive element on the X chromosome (Xd). This skew of the sex ratio is associated with the degeneration of the Y- spermatids in male carriers of Xd. Meiotic drive intensity is decreased in Y linked autosomal factors. In C. dalmanni, the resistant Y chromosome reduces the intensity of the meiotic drive and can reverse the direction. Resistant Y chromosomes (Ym) when paired with Xd cause the transmission of predominantly Y sperm (Presgraves et al., 1997).

In the fruit fly, Drosophila simulans, an X linked meiotic drive has been reported. Males that are carriers of X linked drive produce an abundance of female offspring caused by a deficiency of Y bearing sperm. Nondisjunction of the Y chromatids during meiosis II results in the failure of nondisjunctioned (YY) spermatids to develop into functional sperm (Cazemajor et al., 2000).

In the mosquito, Aedes aegypti and Culex pipiens, meiotic drive is found on chromosome I. In Ae. Aegypti, the driver gene (D) is linked to the male determining allele (M); the responder locus is linked to the female determining allele (m) on the homologous chromosomes. If the drive is linked to the female determining allele and responder liked to the male determining allele it causes the genes to be non-responsive. Heterozygous males which have the driver (M³) and sensitive responder (m⁸) causes the
fragmentation of the sensitive responder (m³) bearing gametes during spermatogenesis. This drive system causes a highly male biased sex ratio (Cha et al., 2006).

Sex ratio distortion in bovine sperm has been found by single sperm typing (Szyda et al., 2000). The majority of bulls (19 of 35) studied showed an excess of X-bearing sperm (53%) than Y-bearing sperm (46%), with the most extreme bulls showing an X-bearing sperm population of 70 to 73%. High distortion rates have been shown to run in families, two of the three bulls with extreme X populations are from the same family. In this study, they looked at the recombination rate of the pseudoautosomal region of the sex chromosome and found a significant skew in the X and Y chromosomes. Two hypotheses were proposed for this deviation. The most straightforward hypothesis was that recombination can produce a certain combination of alleles that are detrimental to Y-sperm viability or preferential to X-sperm viability. Y recombinant products were being lost at some stage during spermatogenesis. The alternative hypothesis was that there may be X specific genes located near the bovine pseudoautosomal region, that when expressed affect the viability of the sperm (Szyda et al., 2000).

Nondisjunction of the sex chromosomes has been shown to exist in cattle but has not been readily documented (Eldridge, 1985). Nondisjunction is caused by failure of chromatids to separate during meiosis (Eldridge, 1985). This condition is comparable to the Klinefelter syndrome (XXY) in humans. It has been found in humans that Klinefelter syndrome originates from nondisjunction during maternal or paternal meiosis (Lorda-Sanchez et al., 1992). In bulls, it is characterized by underdevelopment of the testicles and the animal is usually sterile (Eldridge, 1985).
In the mouse, deletions on the Y-chromosome long arm (MSYq) leads to the up-regulation of multiple X- and Y-linked transcripts in spermatids. These expressed genes come from the sex chromosomes and not the autosomal chromosomes, which indicates the loss of specific repressors of the X and Y (gonosomal) transcripts in post-meiotic cells. Along with a known skew in the sex ratio in favor of the female from MSYqdel fertile males, this suggests the presence of an intragenomic conflict between X- and Y-linked genes. Two suspected genes are the X-linked multi-copy gene Xmr and its counterpart MSYq-linked Sly, which are up- and down-regulated, respectively, in the testes of MSYqdel males (Ellis et al., 2005).

A study to investigate the effects of deletions of the mouse Y-chromosome long arm on sperm function by intracytoplasmic sperm injection (ICSI) found that when ICSI was performed the sex ratio was no longer present. Sperm counts in affected animals were lower than control animals. To investigate if sperm carrying the defective Y chromosome were eliminated during maturation, fluorescent in situ hybridization (FISH) was conducted to genotype the spermatozoa directly; this did not provide satisfactory results. With the results obtained by fertilization with ICSI they concluded that deletions on the Y-chromosome do not interfere with the production of Y-chromosome bearing gametes, as judged from the transmission to the offspring (Ward and Burgoyne, 2006).

**Alleles and their Interactions**

Alleles are alternative forms of the same gene, which do not always show simple relationships between dominance and recessiveness (Purves et al., 1998). An allele may produce many phenotypic expressions. Incomplete dominance between alleles produces an intermediate phenotype, which is somewhere between the phenotypes of the parents.
Alleles are genes that code for production of specific proteins or enzymes. Alternate alleles code for alternative forms of the protein or enzyme, the different forms often have different degrees of catalytic activity. The degree of catalytic activity is expressed in the degree of phenotypic expression. For an allele to be dominant, the single copy of the allele must produce enough of its protein or enzyme to give the maximal phenotypic response. An allele with multiple phenotypic effects is called pleiotropic. The environment also plays a part, with variables such as light, temperature, and nutrition. These variables can affect the translation of the genotype into phenotype (Purves et al., 1998).

**Fluorescent In Situ Hybridization**

Fluorescent in situ hybridization (FISH) is used to paint specific chromosomes or genes, which can then be visualized, through a microscope. First the probe for a specific sequence on a gene or chromosome must be made. The probe is made with fluorescent molecules or antigenic sites which fluorescent antibodies can bind. The probe is then used to hybridize to the specific target gene or chromosome. The sample along with the hybridized probe is exposed to an ultra violet light. The light is used to excite the probe by absorption of photon energy, which increases its energy state. This process is called excitation; the probe cannot exist at this state for an extended period of time. The decrease in energy and return of the fluorophore to ground state results in a release of light energy at a lower light wave length. This is called fluorescent emission, and is characterized by Stokes shift where the light energy is released at a longer wave length than which it is excited by. The fluorescent process is cyclic and can be excited
repeatedly. Detection of the signal can be captured by a digital camera, and analyzed by a computer program (Swiger and Tucker, 1996).

Fluorescent in situ hybridization has been used to validate the sex of spermatozoa, embryos and many other diagnostic studies. T. Revey et al. (2004) studied the head area measurements of dead, live, X- and Y-bearing bovine spermatozoa to determine if head area could be used to determine the sex of the spermatozoa. In this study fluorescent in situ hybridization was used to determine the sex of spermatozoa (Revay et al., 2004).

The use of flow cytometry has proven to be a successful process of separating semen into X- and Y- bearing spermatozoa based on the total cellular DNA content. The use of fluorescent in situ hybridization was used to validate this technique (Kawarasaki et al., 1998) (Rens et al., 2001).

In a study to investigate the effects of deletions of the mouse Y-chromosome long arm on sperm function by intracytoplasmic sperm injection (ICSI); fluorescent in situ hybridization was attempted to genotype spermatozoa. FISH did not provide satisfactory results and was unpublished (Ward and Burgoyne, 2006).

Roelofs et al. (2006) used FISH to sex seven day old embryos. In this experiment they investigated time of insemination relative to ovulation to see if a skew in the sex ratio would result.

In situ competition between protamine and fluorochromes for sperm DNA has been described (Bizzaro et al., 1998). In this study, they investigated the relationship between the level of protamines in mouse and human sperm DNA vs. the level of fluorochromes able to bond. Various concentrations of protamines and fluorochromes in standing solutions were used to establish the level of fluorescence in response to the level
of protamines, showing that a competition exists between fluorochromes and protamines for DNA binding. Histones are replaced by protamines during nuclear compaction, which makes the compacted spermatid or spermatozoa unavailable for staining.
MATERIALS AND METHODS

Tissue Preparation for Fluorescent In Situ Hybridization

Fixation was the first step in the process of tissue preparation. The purpose of fixation is to preserve the tissue permanently in as close to a life-like state as possible. Fixation should be carried out as soon as possible after the tissue is removed from the body to keep postmortem changes to a minimum (Humason, 1979). Bovine testicular biopsies were fixed by immersion, which involved taking a 3 to 5 mm piece of tissue and putting it into a labeled cassette, then immersing it into the fixative. The volume of the fixative was approximately around ten times the volume of the tissue. The fixative used in this study due to the quality of the nuclear detail was Bouin’s fixative. Bouin’s fixative is a solution of three fixatives picric acid, formalin, and glacial acetic acid. Picric acid fixes by way of an unknown mechanism, which works well with nuclear detail (Humason, 1979). Formalin fixes by forming cross-linkages between lysine residues in proteins. These cross-linkages do not harm the structure of the proteins being fixed. Acetic acid is one of the oldest fixatives; it is efficient at fixing the nucleus and has rapid penetrating ability. Acetic acid does not cause hardening of the tissue but does cause some swelling. After the tissue had been fixed, the excess fixative was washed out of the tissue to prevent interference in subsequent processes. Bouin’s fixative was washed in 50% alcohol due to the loss of soluble picrates if washed in water (Humason, 1979).

After fixing the tissue it was ready for processing. The processing technique involved removing the water from the tissue and replacing it with paraffin. The first step was dehydration, which was done through a series of graded alcohols, usually 70-100%. The process has to be slowed to prevent distortion of the tissue as it was dehydrated.
Once dehydration was complete a clearing agent was applied. This step removed the alcohol and replaced it with a clearing agent like xylene. Clearing agents caused the tissue to appear translucent. These agents must be compatible with paraffin. The final step was the infiltration of the tissue with paraffin, which supported the tissue. (Humason, 1979)

Once the tissue was infiltrated with paraffin it was embedded. This was performed by placing the tissue in a mold and pouring the same type of paraffin which was used to infiltrate the tissue. The molds were then chilled quickly, to harden the paraffin. Finally the molds were removed (Humason, 1979).

Sectioning of the paraffin blocks were performed by mounting them to the microtome. The top layer was trimmed away then the tissue was sectioned to 5 microns. The tissue section was then floated in a water bath. Gelatin was added to the water bath to help the tissue adhere to the slide. The slide was used to pick up the section then drained of excess water drained and then slide was allowed to dry on a warming tray. After the slides were dry they were ready to be stained with the appropriate protocol (Humason, 1979).

**Fluorescent In Situ Hybridization Protocol**

Fluorescent In situ Hybridization of paraffin embedded testicular tissue was performed by way of a two day protocol. This protocol is the combination of two protocols, STAR-FISH™ Fluorescent In Situ Hybridization in Paraffin-Embedded Tissue Sections and Bovine X-Y sex test for bovine sperm (CA-1611) Day 2. (Cambio Ltd. The Irwin centre Scotland road, Dry Drayton, Cambs CB3 8AR United Kingdom). On day one, slides mounted with tissue sections were removed from the incubator after drying
over-night at 37°C. Slides were de-waxed in xylene three times for five minutes each. Slides were then rehydrated through graded alcohols to water; ‘absolute’ ethanol and distilled de-ionized water were used to prepare 95%, 80%, 60% and 30% alcohols. Slides stayed in the 95, 80, and 60% solutions for 2 minutes each and in 30% for 5 minutes. Slides were next incubated in sodium thiocyanate solution (16g sodium thiocyanate dissolved in 200ml purified water) for ten minutes at 80°C. The temperature in all of the solutions was extremely important. The temperature of the solutions in the Coplin jar was checked, not the temperature of the water bath. Slides were then washed in phosphate–buffered saline solution (PBS) for two minutes. Next the slides were incubated in pepsin solution (0.2g pepsin dissolved in 50ml of 0.1M HCl just before use) for ten minutes at 37°C. After pepsin, the slides were quenched in glycine solution (0.4g glycine dissolved in 200ml double concentrated PBS (2mg/ml)). Slides were washed in PBS for two minutes. The slides were then re-fixed in paraformaldehyde solution (2g paraformaldehyde dissolved in 50ml of PBS at 80°C, cooled to room temp before use, used on day of preparation.) for two minutes. Slides were washed well in PBS three times for five minutes each. The slides were then dehydrated through graded alcohols, and then air dry. The chromosome paints (X-Y FISH Bovine Sex Test Kit X-Biotin Y-Cy3 labeled) were warmed to 37°C, followed by vortexing and centrifuging them for 1-3 seconds. The probes were denatured for 10 minutes at 65°C, and held at 37°C for 30-60 minutes. Ten µl of the denatured paint were applied to the center of the sample, and the sample was covered with a glass coverslip (22x40mm) and sealed with rubber cement. The slide was placed into The GeneMachines® HybChamber™, and the sealed slides
were denatured at 78°C for ten minutes. The slide was placed horizontally in humidified incubator to hybridize overnight at 37°C.

On day 2, the following solutions: two Coplin jars of stringency wash, two Coplin jars of solution 1XSSC, and one Coplin jar of detergent wash solution, were pre-warmed to 45°C in water bath at least 30 minutes before starting the protocol. The slides were taken out of the incubator and HybChamber™, and placed into solution 1XSSC (25ml of 20XSSC diluted into 475ml of double distilled water) for five minutes. The rubber cement and cover slip were removed from the slides. The slides were not allowed to dry. Slides were washed twice in stringency wash solution (25ml de-ionized Formamide into 25ml of 2XSSC) by incubating them for five minutes each time at 45°C. The slides were washed twice by incubating in 1XSSC for five minutes each time at 45°C. Slides were then incubated three times for four minutes in detergent wash solution (250µl of detergent DT diluted into 500ml 4XSSC) at 45°C. The biotin (FITC) detection kit (1089-KB-50) was used, 100µl of working reagent D were applied to the slide and covered with Parafilm™ immediately. Working reagent D was a mix of the supernatant of working reagent B (.30µl of detection reagent B1 in 61.75µl of working reagent A) and working reagent C (.6µl of detection reagent B2 in 61.5µl of working reagent A). Working reagent A was 19µl Blocking protein BP in 106µl Detergent wash solution. The slides were incubated in a humidified incubator for 20 minutes at 37°C. After the incubation was complete the Parafilm™ was removed and the slides were then washed three times in the Detergent wash solution at room temperature for four minutes per wash. The slides were drained well and mounted with 50µl of reagent MD (mountant (antifade) + DAPI). Finally a glass coverslip was applied to the slide and sealed with nail varnish. The slides
were stored in the dark at 4°C. Slides were viewed under fluorescent microscopy using standard epifluorescence filters for FITC and Cy3.

**Fluorescence Microscopy**

Seminiferous tubules from testicular biopsies were observed with phase optics (40X NeoFluar Ph2 objective) on a research microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epi-illumination for fluorescence microscopy. Fluorescence microscopy was achieved by exposure of the sample to UV light generated by a 50 W mercury arc lamp. The UV light was passed through a FITC filter set (exciter BP 450-490, dichromatic beam splitter FT 510, barrier LP 520) and TRITC/Dil filter set (exciter D540/25, beam splitter 565dclp, emitter D605/55). Digital images were taken with a Nikon coolpix 4500 camera mounted to the eye piece with a LensPlus LE-Adapter. The camera was set on museum; the pictures were taken one at a time by changing from the FITC filter set to the TRITC filter set and phase between pictures. Pictures were downloaded from the camera to an Emachines® computer. Pictures were then transferred into computer program, Adobe Photoshop, where the color of the picture was enhanced (Naughton et al., 2006). FITC and Cy3 pictures were adjusted in color balanced. Cy3 pictures, once in color balance, were adjusted by changing shadows: cyan to 100%, then adjusting highlights: red to 95%, green to 70%, and blue to 55%. FITC pictures, once in color balance, were enhanced by adjusting shadows: cyan to 100%, magenta to 100%, and yellow until the cell was clearly visible. The highlights were then adjusted: red to 100%, green to 100%, and blue to 8-15%. Next the midtones were fine tuned. The last step of the procedure was to copy the background and then delete the locked background. The background opacity was adjusted between 30-70% until both labels were clearly
visible. The pictures were then transferred to Image Tool (Chandler et al., 2002). The pictures were analyzed using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from (ftp://maxrad6.uthscsa.edu)) (Chandler et al., 2002).

**Statistical Methods and Calculations**

The data were analyzed using the general linear model in the SAS program version 9.1. (SAS Institute Inc., 2002-2003)

The model included the following:

\[
Y_{ijkl} = \mu + Sex_i + Slide_j + Picture-set_k + Tubule(Slide)_{j(l)} + Sex*Slide_{ji} + Sex*tubule(slide)_{j(l)l} + \text{random error}_{ijkl}
\]

Where:

- \(Y_{ijkl}\) = objective
- \(\mu\) = overall mean
- \(Sex_i\) = fixed effect of Sex (X or Y)
- \(Slide_j\) = fixed effect of Slide (1, 2, 3, 4, or 5)
- \(Picture-set_k\) = fixed effect of Picture set (1 or 2)
- \(Tubule(Slide)_{j(l)}\) = random effect of the Tubule 1 nested within Slide j
- \(Sex*Slide_{ji}\) = interaction between Sex i and Slide j
- \(Sex*tubule(Slide)_{j(l)l}\) = interaction between Sex i and Tubule 1 nested within Slide j and
- \(e_{ijkl}\) = random residual
The model effects were tested with the appropriate error terms by using the random statement with the test option as required by expected means squares as follows: (Steel and Torrie, 1980).

\[
\begin{align*}
\text{sex} &= \text{Variance (Error)} + \text{Quadratic form (sex, sex*slide, sex*tubule(slide))} \\
\text{slide} &= \text{Variance (Error)} + 4 \text{Variance (tubule(slide))} + \text{Quadratic form (slide, sex*slide, sex*tubule(slide))} \\
\text{Picture-set} &= \text{Variance (Error)} + \text{Quadratic form (picture-set)} \\
\text{tubule(slide)} &= \text{Variance (Error)} + 4 \text{Variance (tubule(slide))} + \text{Quadratic form (sex*tubule(slide))} \\
\text{sex*slide} &= \text{Variance (Error)} + \text{Quadratic form (sex*slide, sex*tubule(slide))} \\
\text{sex*tubule(slide)} &= \text{Variance (Error)} + \text{Quadratic form (sex*tubule(slide))}
\end{align*}
\]
RESULTS

Fluorescent in situ hybridization was used to visualize and determine the sex ratio of the round spermatid population within seminiferous tubules as seen in Figures 1 and 2. Figure 1 (A, B, C) shows a seminiferous tubule labeled by fluorescent in situ hybridization to illustrate chromosome labeling and cell types, (A) Cy3 labeled Y-chromosome (red), (B) FITC labeled X-chromosome (green) (C) combined pictures of A and B, (D) a phase contrast picture of the same tubule, (E) Hemotoxylin and Eosin stained tubule from the same tissue, but not the same tubule. Each picture has cell types denoted. Figure 2 depicts a tubule with a skew in the sex ratio. (A) Cy3 labeled Y-chromosome (red), (B) FITC labeled X-chromosome (green), (C) combined pictures of A and B to evaluate sex ratio, (D) phase contrast picture to validate tissue. The cell types can be distinguished by their relative size and position within the tubule along with the number of sex chromosomes within each cell. The photographs were used to determine the sex ratio of each tubule.

Analysis of variance on X- and Y-chromosome bearing round spermatids as affected by sex, slide, picture set, and tubule is presented in Table 1. Sex demonstrated that the variation in round spermatid sex ratio differed between slides (P=0.0001) in the overall study. Sex by slide interactions were shown to be non-significant (P = 0.0815). Sex within tubule nested in slide showed a significant deviation in sex ratio (P=0.0122). Picture set showed no significant difference in counts, confirming that the counts were consistent (P=0.2096).
**Figure 1.** Fluorescent in situ hybridization of paraffin embedded seminiferous tubule. (A) Shows the Y-chromosome labeled with Cy3 (Red). (B) Shows the X-chromosome labeled with biotin FITC (Green). (C) Shows picture A and B combined; this allows the identification of cell types and sex of spermatids to establish the sex ratio of this piece of the tubule. (D) Phase contrast picture to verify tissue. (E) Hemotoxylin and Eosin (H&E) picture from the same tissue. Scale bar = 20 µm.
C. Spermatogonia

X spermatid

Y spermatid

Spermatocyte

D. Spermatid

Spermatocyte

Spermatogonia
E.

- Spermatogonia
- Spermatocyte
- Spermatid
Figure 2. Pictures of a seminiferous tubule with a significant skew in favor of the Y-chromosome. (A) Y-chromosome labeled with Cy3 (Red arrow). (B) X-chromosome labeled with Biotin FITC (Green arrow). (C) Combination of picture A. and B. showing the sex chromosome or chromosomes in each cell. (D) Phase picture to verify tissue. Scale bar = 20 μ
Table 1. Analysis of variance of percent X and Y-chromosome bearing round spermatids as affected by sex, slide, picture set and tubule

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Total</td>
<td>243</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>627.13</td>
<td>19.07</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Slide</td>
<td>4</td>
<td>1304.94</td>
<td>39.68</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Picture set</td>
<td>1</td>
<td>52.33</td>
<td>1.59</td>
<td>0.2096</td>
</tr>
<tr>
<td>Tubule (Slide)</td>
<td>56</td>
<td>398.65</td>
<td>12.12</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Sex * Slide</td>
<td>4</td>
<td>69.96</td>
<td>2.13</td>
<td>0.0815</td>
</tr>
<tr>
<td>Sex * Tubule (Slide)</td>
<td>56</td>
<td>54.02</td>
<td>1.64</td>
<td>0.0122</td>
</tr>
<tr>
<td>Residual</td>
<td>121</td>
<td>32.89</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R² (%) 0.888077

Count Mean 23.91

Figures 3 to 7 show the counts of X and Y-chromosome bearing spermatids vs. tubule within slide. In Figure 3, only tubule 1 fell outside of the error bars with a skew toward the Y-chromosome, which is 10% of the slide. Figure 4 had three tubules, 7, 13 and 10, were significantly skewed toward the Y-chromosome, which was 20% of the slide. Figure 4 was the only one which had a tubule (#4), which was significantly skewed toward the X-chromosome; this corresponded to 6% of the slide and 1.6% overall all tubules. In Figure 5, tubule 4 was skewed toward the Y-chromosome, which was 9% of the slide. Figure 6 had two tubules, 9 and 15, that were skewed toward the Y-chromosome, this related to 13% of the slide. Figure 7 had the largest percentage of skewed tubules, with tubules 2, 4, and 9 showing a significant deviation from 1:1 which related to 30% of the slide. Overall 10% of all of the tubules were significantly skewed to the Y-chromosome. Figure 4 showed the only significant tubule skewed toward the X-chromosome, which equaled 6% of that slide’s tubules and 1.6% of the overall.
Figure 3. Comparison of X and Y round spermatid counts with error bars per tubule in slide 1, (*) indicates tubule with significant difference (P < 0.05) in sex ratio.

Figure 4. Comparison of X and Y round spermatid counts with error bars per tubule in slide 2, (*) indicates tubule with significant difference (P < 0.05) in sex ratio.
**Figure 5.** Comparison of X and Y round spermatid counts with error bars per tubule in slide 3, (*) indicates tubule with significant difference (P < 0.05) in sex ratio.

**Figure 6.** Comparison of X and Y round spermatid counts with error bars per tubule in slide 4, (*) indicates tubule with significant difference (P < 0.05) in sex ratio.
Figure 7. Comparison of X and Y round spermatid counts with error bars per tubule in slide 5, (*) indicates tubule with significant difference (P < 0.05) in sex ratio.

Table 1 showed sex within slide to be non-significant (P = 0.0815). However as seen in Table 2 and Figure 8, the skew existed in four out of the five slides. In Figure 8 slides 2, 3, 4, and 5 the sex ratio was skewed toward the Y-chromosome, in slide 1 the count was well within the standard error but did trend toward the X-chromosome.

Table 2. Least squares means of X and Y-chromosome bearing spermatids per slide and the direction of sex ratio skew.

<table>
<thead>
<tr>
<th>Slide</th>
<th>Green (X)</th>
<th>Red (Y)</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.5 ± 1.2</td>
<td>19.9 ± 1.2</td>
<td>even</td>
</tr>
<tr>
<td>2</td>
<td>28.3 ± 1.0</td>
<td>31.0 ± 1.0</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>18.3 ± 1.2</td>
<td>21.3 ± 1.2</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>17.8 ± 1.0</td>
<td>22.4 ± 1.0</td>
<td>Y</td>
</tr>
<tr>
<td>5</td>
<td>25.9 ± 1.2</td>
<td>32.3 ± 1.2</td>
<td>Y</td>
</tr>
</tbody>
</table>
Figure 8. Comparisons of X and Y Least square means with error bars per slide.
DISCUSSION

Studies have been conducted for many years on the degeneration of cells during spermatogenesis. Theoretical yields of 64 spermatids per A-spermatogonia have been reported by Amann (1962) due to degeneration during spermatogenesis, although in his study there was no report of spermatid degeneration. Roosen-Runge (1955), reported a 10% degeneration of young spermatids in rats. Oakberg (1956) found a 13% loss during the two meiotic divisions in mice. Previous studies have shown that skews in the sex ratio of ejaculated semen do exist and have been shown to exist in wave patterns (Chandler et al., 2002). These wave patterns fluctuate from 13.5 days and also at approximately 42 days. Further investigation to see if the skew comes from the production of spermatozoa was indicated (Chandler et al., 2002).

The purpose of this study was to determine if a skew in the sex ratio occurred in the round spermatid population. Fluorescent in situ hybridization was used to visualize the sex chromosomes in round spermatids from bovine testicular biopsies. The count of the X and Y chromosomes was used to determine the sex ratio of the round spermatids. The ratio of X-bearing to Y-bearing spermatids in our study did not equal to 1:1, this implied that there is degeneration of spermatids which may result in a skew of the sex ratio.

Different sources of skewing have been discussed in various studies whether it comes from nondisjunction in meiosis II as in the Drosophila (Cazemajor et al., 2000), meiotic drive systems which caused fragmentation of the gametes during spermatogenesis as in the mosquito (Cha et al., 2006), or degeneration of Y spermatids as in the Stalked eyed flies (Presgraves et al., 1997).
In the mouse, Ellis et al. (2005) showed that deletions on the mouse Y long arm lead to the up-regulation of multiple X- and Y-linked transcripts in spermatids, along with a sex ratio skew in favor of females. This suggests the existence of an intragenomic conflict between X- and Y-linked genes. Ward and Burgoyne (2006) contested these results by using ICSI. In this study they found that when ICSI was performed the sex ratio disappeared, and concluded that the Y-chromosome bearing spermatozoa lost ability to fertilize in vitro. Fluorescent in situ hybridization was attempted to genotype the spermatozoa’s sex ratio but was unsuccessful. This limited their ability to determine the actual sex ratio of the semen.

The results of our study agree with the concept of meiotic drive which causes a skew in the round spermatid population. Skews in bovine sex ratios per ejaculate have been shown to exist with the use of polymerase chain reaction (Chandler et al., 1998). Szyda et al. (2000) also showed a skew in sex ratio per ejaculate with the use of single sperm typing. Recombination rates in the pseudoautosomal region of X and Y bearing chromosomes have been correlated with the sex ratio distortion in bovine sperm. Their hypothesis was that recombination can produce a certain combination of alleles that are detrimental to the Y sperm viability or preferential to X sperm viability. An alternate hypothesis proposed that X specific genes located close to the bovine PAR which are expressed in the spermatids can affect the viability of the sperm (Szyda et al., 2000). The results of our study showed a skew already present at the level of the round spermatids pointing to early degeneration of spermatids which resembles a meiotic drive; this would have an effect on the spermatid population and not the mature sperm. Also our data point toward a skew in the Y-chromosomes direction. As we evaluated percent tubules per
slide, the number of seminiferous tubules that varied from 50:50 toward the Y-chromosome was 9 to 30% per slide, with an overall average of 10%. Table 3, slide 2 shows that the skew can be reversed in tubules. In tubule 4 there is a significant skew toward the X-chromosome. Also in figure 8, the spermatid counts per slide show a significant skew with slides 2, 3, 4, and 5 in favor of the Y-chromosome. Also in Figure 8, although not significant, slide 1 shows a reversal with the counts leaning toward the X-chromosome. This indicates at two different levels with non corresponding slides, that the skew can be reversed. The majority of the skew is toward the Y-chromosome. One hypothesis would imply the presence of a meiotic drive due to the observation of a one way skew and also the presence of a modifier due to the reversal of the skew. Another hypothesis is that this correlates with Chandler et al. (2002) where it was shown that waves fluctuate at percentages above 50% in the direction of the Y-chromosome. These researchers reported the frequency at which the skew fluctuates over time was demonstrated. In the present study the source of the skew was shown. Although we cannot say for certain that there is a meiotic drive, our study did show a definite sex ratio skew at the level of the spermatid.

Fluorescent in situ hybridization has proven to be a challenging process. The inefficiency in this process should be noted; in three sets of ten tests only five tests have yielded any data. In the five tests that worked, all came from the same tissue. In one other test that worked the tissue was to blame in that no haploid cells were available for the collection of data. Hemotoxylin and Eosin (H&E) stained slides taken from the tissue used in these tests showed that the tissue had normal morphology. The study of this tissue required the combination of protocols due to the lack of a set protocol for this tissue.
Chromosome paints intended for denatured sperm were integrated into a paraffin embedded tissue protocol in an attempt to sex spermatids. Further work in establishing a set protocol which will consistently provide usable data is necessary.
CONCLUSIONS

Skews in sex ratios have been the subject of much speculation for many years. Scientists have debated whether sex ratio skews exist and where it would originate. It has been widely thought that skews in sex ratios in mammalian sperm do not exist, with each ejaculate the ratio would be 50:50. Chandler et al. (1998) used PCR to demonstrate that skews in sex ratio do exist between ejaculates within bulls. Szyda et al. (2000) used single sperm typing to expand this theory to demonstrate that not only do skews exist but also run in families. Chandler et al. (2002) reported a sin wave which resembles a wave given by hormonal positive and negative feedback systems (Chandler et al., 2002). This system would relate to modifiers having the same positive or negative control over drive phenotype response, which is how the body regulates itself. The evidence points toward a meiotic drive along with a modifier which regulates the phenotypic expression of the drive to produce a sex ratio.

In the present study fluorescent in situ hybridization was used to determine if a sex ratio skew was present in the round spermatid population. These findings show that the skew does exist and occurs as early as the round spermatid population. The data show a 10 – 30% variation in the number of seminiferous tubules with a significant skew per slide, which can be compared to the percentage of the skew found in the testicle. Most of the skew is toward the Y-chromosome, but in slide 2 tubule 4 a significant skew was shown toward the X-chromosome. This skew toward the X-chromosome indicates that the skew can be counteracted and even reversed. Table 2 also shows a skew with slides 2, 3, 4, and 5 leaning toward the Y-chromosome, while slide 1 fell within the error bars but leaned toward the X-chromosome.
Although we cannot prove that a meiotic drive exists in bovine. Our findings agree with the theory of a meiotic drive existing in bovine, with drives pushing the population in one direction and modifiers counteracting or reversing their effects. Along with the possible effects of the environment influencing the drive, such as crowding effect brought on by intense group housing. Further investigation is necessary to completely understand this phenomenon and what factors affect it.
LITERATURE CITED


APPENDIX

FISH Paraffin Sections Protocol

Day 1

Note: Denature Probe 30-60 min ahead of time

1. Collect 3-6 micron tissue sections on coated slides, dry over night @ 37°C.

2. Dewax in Xylene, 3 times for 5 min. each.

3. Rehydrate through graded alcohols to water 95, 80, 60, and 30% for 2 min. For all except 30% which is 5min.

4. Incubate in Sodium Thiocyanate solution for 10 min. @ 80°C. (Care!!)

5. Wash in PBS for 2 min.

6. Incubate in Pepsin solution for 10 min. @ 37°C.

7. Quench the Pepsin in Glycine solution.

8. Wash in PBS for 2 min.


10. Wash well in PBS: 3 changes over 15 min.

11. Dehydrate through graded alcohols for 2min in 30, 60 & 80%, for 5 min. in 95%. Air dry.

12. From protocol CA-1611 steps 24, 25.

- Warm chromosome paints to 37°C, vortex & centrifuge for 1-3 sec.
- Denature probe for 10 min @ 65°C, hold @ 37°C for 30-60 min.

13. Apply 10-15 µl of paint mix to the center of the slide.

14. Cover with glass coverslip (22 x 40mm) and seal with rubber cement.

15. Denature the sealed slide @ 78°C for 10 min.

16. Place the slides horizontally in a humid chamber and hybridise overnight @ 37°C
Day 2

Prepare working reagent D.
Pre-warm to 45°C in water bath at least 30 min. before starting:
  - Two Coplin jars of Stringency wash solution
  - Two Coplin jars of solution 1X SSC
  - One Coplin jar of Detergent wash solution

**Note:** Temperature is important. Check the temperature of the solutions in the coplin jars, not the water in the water bath.

17. Take the slide out of the incubator and leave in solution 2X SSC for 5 min. Take off rubber cement and leave in solution 2X SSC to remove the coverslip.
   **Note:** Do not allow the slide to dry

18. Wash slides twice by incubating for 5 min. each in stringency wash solution @ 45°C.

19. Wash slides twice by incubating for 5 min. each in 2X SSC @ 45°C.

20. Incubate slide for 4 min. in detergent wash solution @ 45°C.

21. Apply 100µl of working reagent D onto the slide and cover with Parafilm™ immediately.

22. Incubate slide in a humidified box for 15-20 min. @ 37°C.

23. Remove Parafilm™ from the slide and wash 3 times for 4 min. in detergent wash solution at room temp.

24. Drain slide well and mount with 10µl of working reagent F.

25. Apply glass coverslip and seal with nail varnish store slides in the dark at 4°C

26. View slides using standard epifluorescence filters for FITC, Cy3 & counter stain DAPI.
FISH Paraffin Section Solutions

1. **20X SSC**
   - 87.6g NaCl
   - 41.1g Na citrate
   - Up to 500ml double distilled (DD) water pH to 7.4 w\ HCl

2. **1X SSC**
   - 25ml 20X SSC
   - 475ml DD water

3. **4X SSC**
   - 100ml 20X SSC
   - 400ml DD water

4. **Detergent Solution**
   - 500ml 4X SSC
   - 250µl Tween 20

5. **Stringency Solution**
   - 25ml Deionised Formamide
   - 25ml 1X SSC

6. **Pepsin Solution**
   - .2g Pepsin
   - 50ml .1M HCl

7. **1 N HCl**
   - 100ml of distilled water
   - 8.33 12N HCl

8. **10mM HCl**
   - 2ml of 1N HCl
   - 200ml of distilled water

9. **Working Reagent A**
   - 22.8µl Blocking Protein
   - 106µl Detergent Wash Solution

10. **Working Reagent B**
    - .30µl Detection reagent B1
      - 61.75µl Working reagent A
      - (Incubate in the dark for 5 min. microcentrifuge at 11,000g for 5min)

11. **Working Reagent C**
    - .6µl Detection Reagent B2
      - 61.5µl Working Reagent A
      - (Incubate in the dark for 5 min. microcentrifuge at 11,000g for 5min)

12. **Working Reagent D**
    - Mix Supernatant of working reagent B & C

13. **Working Reagent F**
    - Mountant, DAPI
14. **PBS 2X** 16g NaCl
   0.4g KCl
   2.38g Na$_2$HPO$_4$
   0.4g KH$_2$PO$_4$
   1000ml Distilled Water

15. **Sodium Thiocyanate** 16g Sodium Thiocyanate
   200ml Distilled Water

16. **Paraformaldehyde Solution** 2g Paraformaldehyde
   50ml PBS
   (Mix at 80°C cool to room temp. use day of
    Preparation Care!)

17. **Glycine Solution** 0.1g Glycine
   200ml 2X PBS

18. **Alcohols** 95, 80, 60, 30% concentrations
VITA

Michael Alan Stout grew up in Erath, Louisiana. His parents are Mr. and Mrs. William Harold Stout, and he has one sister and two brothers. His sister is Tellie Stout, who is a surgical scrub nurse at Abbeville General Hospital. He has two brothers, Robert and John. Robert is a nursing student at UL at Lafayette. John is an air-conditioning technician for Butcher Air Conditioning in Lafayette. Michael attended public school in Erath and was an active 4-H member, showing Red Brangus cattle. He was also an officer in the United Red Brangus Association.

Michael attended the University of Louisiana at Lafayette from 1999-2004. While at ULL, he worked on the Desoto Cattle Project with Dr. Terry Clements. The Desoto Cattle Project tested parasite resistance. Michael also worked on a water quality project at the ULL Cade Farm with Dr. Lora L. Goodeaux. Michael was a member of Alpha Zeta honor society, serving as treasurer for the 2002-2003 school year.

Michael attended Graduate School at LSU from 2005-2006. He studied Reproductive Physiology under Dr. John E. Chandler in the Department of Dairy Science. While attending LSU, Michael worked in various capacities at Genex Cooperative.