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CHANGES IN THE PROPORTION OF X- AND Y-CHROMOSOME BEARING SPERM ATTACHED TO OVIDUCTAL EPITHELIAL CELLS OVER TIME

By

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ABSTRACT

CHANGES IN THE PROPORTION OF X- AND Y-CHROMOSOME BEARING SPERM ATTACHED TO OVIDUCTAL EPITHELIAL CELLS OVER TIME

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Recent data indicate that increasing the time from insemination to ovulation inversely affects the subsequent sex ratio of calves (Pursley, et al., 1998, Macfarlane, 2003). Differences in X and Y sperm that allow x sperm to survive longer is one possible explanation for this phenomenon. The objectives of the current study were (1) to develop and validate a real-time PCR based assay for the quantification of Y-chromosome bearing sperm and (2) to use this assay to determine if the proportion of Y sperm that remain attached to cultured oviductal cells deviates over time. To develop a quantitative real-time PCR assay, primers were designed to amplify a portion of the SRY gene and a section of autosomal DNA. Standard curves were developed from plasmids containing the cloned fragments of the SRY gene and the 1.715 satellite region. Sperm were co-incubated with *in vitro* cultured oviductal cells for 2h, 12h, 24h, or 36h. Sperm that remained attached to the oviductal cells after each time-point were removed and percent Y sperm for each sample was determined using the assay. Sperm attached to oviductal cells consisted of 41.01 ± 1.75 % Y sperm at 2h, 48.37 ± 1.89 % Y sperm at 12h, 33.59 ± 1.49 % Y sperm at 24h, and 41.30 ± 1.59 % Y sperm at 36h (mean ± SEM). Differences between time points were only significant for 12h versus 24h ($P < 0.05$), indicating more Y sperm became detached during this time.

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
bp	Base Pair
BSA	Bovine Serum Albumin
BSP	Bovine Seminal Plasma
C0	Starting Copy Number
Ca^{2+}	Calcium
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
cDNA	Complementary DNA
CO_2	Carbon Dioxide
C_T	Cycles to Threshold
CV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triposphate
DTT	Dithiothreitol
D-PBS	Dulbecco's Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
g	Gravity
h	Hour

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
H-Y	Histocompatibility Y
HDL	High Density Lipoprotein
IPTG	Isopropyl-beta-D-thiogalacto pyranoside
IVF	*In Vitro* Fertilization
KCl	Potassium Chloride
LB	Luria Bertani Broth
LSD	Least Significant Difference
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
$MgCL_2.6H_2O$	Magnesium Chloride Hexahydrate
min	Minute
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaH_2PO_4	Sodium Phosphate Monobasic
PB	Polar Body
P:C:I	Phenol:Chloroform:Isoamyl Alcohol
PCR	Polymerase Chain Reaction
R^2	Regression Coefficient
s	Second
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SRY	Sex-Determining Region of the Y Chromosome

TALP	Modified Tyrode's Albumin with Lactate and Pyruvate
TE	Tris-EDTA
TrisHCL	Tris-Hydrocloride
UTJ	Utero-Tubual Junction
Xgal	5-Bromo-4-Chloro-3-Indolyl-beta-D-galactopyranoside
X sperm	X-Chromosome Bearing Sperm
Y sperm	Y-Chromosome Bearing Sperm

INTRODUCTION

Predetermination of the sex of livestock could increase efficiency of agricultural production, reduce animal production costs and provide a means to control and eliminate undesirable or lethal sex-linked traits (Gledhill, 1988, Windsor, et al., 1993, Hossain, et al., 1998, Rorie, 1999, Seidel and Johnson, 1999). The sex chromosome of the fertilizing spermatozoon determines the sex of mammalian offspring (Painter, 1923, Painter, 1924). X-chromosome bearing sperm (X sperm) produce females and Y-chromosome bearing sperm (Y sperm) produce males (Painter, 1923, Painter, 1924). Interest in controlling the sex of livestock led to investigations of differences in X and Y sperm, methods to detect these differences, and methods to separate sperm based on these differences. Physiological mechanisms that may alter sex ratio have also been of interest and include facilitating or inhibiting transport of either X or Y sperm, preferential selection of sperm at fertilization, or sex-specific embryo death (Pursley, et al., 1998, Rosenfeld and Roberts, 2004). Increasing the interval between insemination and ovulation was found to alter subsequent gender ratio in favor of female calves (Pursley, et al., 1998, Macfarlane, 2003).

X and Y sperm can be distinguished based on differences in DNA content or presence of specific gene sequences on the X or Y chromosome. There are a handful of techniques that can use these differences to quantify X and Y bearing sperm in a research or commercial setting. These methods include karyotyping, in situ hybridization and fluorescence in situ hybridization (FISH), polymerase

chain reaction (PCR), and flow cytometric measurement of DNA content. While these approaches are accurate, they are limited by high costs, low yield, technical difficulty and the requirement of specialized equipment. These methods are discussed in detail in Chapter One. The work described in Chapter Two focuses on the development of a new real-time PCR assay for quantification of Y sperm in cattle.

After insemination, sperm travel from the site of insemination, through the female reproductive tract, and form a reservoir in the oviductal isthmus (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984, Wilmut and Hunter, 1984). The oviductal sperm reservoir appears to have four functions. First, it may prevent polyspermic fertilization by reducing the number of sperm that reach the oocyte at a time (Hunter and Leglise, 1971). Second, the oviductal sperm reservoir maintains sperm fertility during the pre-ovulatory period (Pollard, et al., 1991). Third, the physiological state of sperm may be regulated by the oviductal sperm reservoir. Membrane contact between sperm and oviductal epithelial cells maintained low intracellular calcium concentration, delayed capacitation, and prolonged viability of sperm (Dobrinski, et al., 1996, Dobrinski, et al., 1997). Finally, the oviductal sperm reservoir selects viable sperm that are uncapacitated, acrosome intact, and morphologically normal (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001). Together, the functions the oviductal sperm reservoir ensure successful fertilization by providing the appropriate number of sperm in the appropriate physiological state for fertilization at the appropriate time (Suarez, 2002, Hunter, 2003). The membrane changes of

capacitation allow sperm to release from the reservoir and fertilize an oocyte (Talevi and Gualtieri, 2001, Therien, et al., 2001). The environment sperm encounter in the female reproductive tract is reviewed in Chapter One.

Increasing the time between insemination and ovulation increases the time sperm must spend in the sperm reservoir (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). Differences in X and Y sperm in their ability to remain attached to the epithelium in the reservoir over long periods could influence the sex ratio of offspring. The work of Chapter Three investigates the ability of X and Y sperm to remain attached to oviductal epithelium over time.

To our knowledge, no studies have been published that test the ability of X and Y sperm to remain bound to oviductal epithelium over time. The overall goal of the present study was to develop a real-time PCR assay for quantification of Y sperm in cattle and to use this assay to detect differences in the proportion of Y sperm that remained bound to *in vitro* cultured oviductal epithelial cells during prolonged co-incubation. Results of the experiments reported in this thesis are novel and important because a reliable high throughput method for quantification of Y sperm will be a useful tool in experiments to answer biological questions about differences in X and Y sperm. Consistent differences between X and Y sperm were found in their ability to remain attached to oviductal cells during prolonged co-incubation.

CHAPTER ONE

A Review of Literature

I. INTRODUCTION

Several studies show that increasing the interval between insemination and ovulation alters subsequent gender ratio in favor of female calves (Vasconcelos, et al., 1997, Pursley, et al., 1998, Macfarlane, 2003). Calf gender is determined by the sex chromosome of the fertilizing spermatozoon (Painter, 1923, Painter, 1924). Upon fertilizing an oocyte, X sperm produce females and Y sperm produce males (Painter, 1923, Painter, 1924). An increase in female calves may be explained by a difference in X and Y sperm that gives an advantage to X sperm when the time sperm spend in the female reproductive tract is increased. Sperm travel from the site of insemination to the oviductal isthmus where they form a reservoir by binding to oviductal epithelium. Increasing the interval between insemination and ovulation increases the time sperm must spend bound to the epithelium in this reservoir (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). Thus, if X sperm have an advantage in remaining bound to the epithelium over extended periods of time they would be favored for fertilization when insemination occurs earlier in relation to ovulation.

The overall hypothesis of this thesis is that X sperm are able to remain attached to oviductal epithelium longer than Y sperm. Differences in the time sperm are able to remain attached to oviductal epithelium may explain differences in gender ratio seen when the time sperm spend in the female

reproductive tract is increased. Testing of this hypothesis requires a reliable, high-throughput method of quantifying X and Y sperm. While methods to identify and quantify X and Y sperm exist, none met the needs of this study. The objectives of my research were: (1) to develop a real-time PCR based assay to quantify Y chromosome bearing sperm and (2) to use this assay to determine if the proportion of X and Y sperm attached to *in vitro* cultured oviductal epithelium changes over time. The objectives of this review are to explain (1) the differences and similarities between X and Y sperm, (2) methods available to identify and quantify X and Y sperm and their limitations, (3) the environment sperm encounter in the female reproductive tract, (4) sperm-oviduct interactions, and (5) what is known thus far about the effect of time of insemination relative to ovulation on X and Y sperm and offspring gender.

II. THE FINAL FRONTIER: LIFE AND DEATH OF SPERM IN THE FEMALE REPRODUCTIVE TRACT

In order to fertilize an oocyte *in vivo*, sperm must travel from the site of insemination to the site of fertilization in the female reproductive tract (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). In cattle, sperm are deposited in the uterus during artificial insemination (AI) and in the vagina during natural mating (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). Fertilization occurs in the ampullary-isthmic junction of the oviduct (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). Sperm must travel through the cervix (in naturally mated animals), into the uterus, through the utero-

tubual junction (UTJ) and into the oviduct (Hawk, 1983). Only a small percentage of the sperm that are inseminated make it to the site of fertilization (Suarez, 1998, Scott, 2000, Suarez, 2002). The process of sperm transport in the female reproductive tract is not just a simple migration from the site of insemination to the site of fertilization (Scott, 2000). It is a complex and dynamic process that includes phases of sperm distribution, the formation of sperm reservoirs, the modulation of sperm physiology and acquisition of fertilization competence, the ascent of competent sperm to the site of fertilization and the elimination of the non-fertilizing sperm population (Scott, 2000).

Sperm transport through the female reproductive tract begins with insemination (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). When natural mating occurs in cattle, 4 to 18 billion sperm are placed in the vagina of the female and must travel through the cervix (Taylor and Field, 1998). When AI is used, the cervix is bypassed and approximately 10 million motile sperm are placed directly into the uterus (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984, Taylor and Field, 1998). For both natural mating and AI, most sperm are lost through retrograde movement out the vagina (Mitchell, et al., 1985). From the site of deposition, sperm must travel to the oviduct to fertilize an oocyte (Hawk, 1983, Hunter and Wilmut, 1983, Hunter and Wilmut, 1984). Primary mechanisms of sperm transport are smooth muscle contractions of the female reproductive tract, ciliary beats, fluid currents, and sperm movement by flagellar activity (Hawk, 1983). Smooth muscle contractions are increased and stronger during estrus, compared with other stages of the estrous cycle (Hawk,

1983). These contractions are primarily responsible for sperm movement through the uterus (Hawk, 1983, Katila, 2001). Once through the uterus, sperm must pass through the UTJ to enter the oviduct. Sperm motility may be necessary for crossing the UTJ (Cooper, et al., 1979, Gaddum-Rosse, 1981). The importance of sperm motility for entering the oviduct was demonstrated by the inability of immotile sperm in crossing the UTJ (Gaddum-Rosse, 1981). Spatial constraints, epithelial surface characteristics, and fluid secretions affect sperm motility (Katz, et al., 1989). Functional interactions between sperm and luminal fluids and epithelial surfaces of the female reproductive tract promote the selection of physiologically normal sperm (Krzanowska, 1974, Mitchell, et al., 1985, Larsson, 1988, Katz, et al., 1989).

During transport, sperm accumulate and are retained in some regions of the reproductive tract for prolonged periods (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984, Wilmut and Hunter, 1984). These regions are referred to as sperm reservoirs (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984, Wilmut and Hunter, 1984). Ligation of oviducts 6 h after mating resulted in the recovery of few fertilized oocytes after ovulation, indicating that competent sperm have not enter the oviducts in sufficient numbers to fertilize oocytes prior to 6 h after mating (Hunter and Wilmut, 1983, Wilmut and Hunter, 1984). Increasing the time between mating and ligation increased the percentage of fertilized oocytes recovered (Hunter and Wilmut, 1983, Wilmut and Hunter, 1984). These results indicate that a population of sperm capable of fertilization is established in the oviduct over a period of "not less than 6 hours and probably more than 12 hr"

(Wilmut and Hunter, 1984). Further experiments showed that competent sperm that have entered the oviduct within 10 to 12 h of mating in sufficient numbers to fertilize oocytes are restricted to the caudal 2 cm of the isthmus for a further 18-20 h (Hunter and Wilmut, 1984). This was demonstrated by ligation of the oviduct approximately 2 cm above the UTJ either prior to or after ovulation (Hunter and Wilmut, 1984). When ligation occurred prior to ovulation, few fertilized oocytes were recovered (Hunter and Wilmut, 1984). These results suggested that viable sperm are sequestered in the caudal isthmus for most of the pre-ovulatory period (Hunter and Wilmut, 1984). Thus, the functional sperm reservoir, the one drawn on at the time of ovulation, is located in the caudal oviductal isthmus (Hunter and Wilmut, 1983, Hunter and Wilmut, 1984, Wilmut and Hunter, 1984). This is considered the functional reservoir because sperm that interact with the oocyte proceed from this reservoir (Hunter and Wilmut, 1983, Hunter, 2003).

A. The Oviductal Sperm Reservoir

The oviductal sperm reservoir ensures successful fertilization by providing the appropriate number of sperm in the appropriate physiological state for fertilization at the appropriate time (Suarez, 2002, Hunter, 2003). The functions of the oviductal sperm reservoir are to prevent polyspermic fertilization, maintain sperm fertility, regulate physiological state of sperm, and select competent sperm (Suarez, 1998, Topfer-Petersen, 1999b, Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Suarez, 2002, Topfer-Petersen, et al., 2002, Gualtieri and Talevi, 2003).

The oviductal sperm reservoir is located in the caudal isthmus of the oviduct (Hunter and Wilmut, 1983). The functional sperm reservoir is found in the isthmus because that is the first region the sperm encounter (Lefebvre, et al., 1995, Lefebvre and Suarez, 1996, Petrunkina, et al., 2001, Fazeli, et al., 2003). When sperm enter the oviduct, they become trapped in the isthmus, forming the reservoir and preventing them from traveling further up the oviduct (Lefebvre, et al., 1995, Lefebvre and Suarez, 1996, Petrunkina, et al., 2001, Fazeli, et al., 2003). The oviduct has narrow channels and thick mucus (Suarez, et al., 1997, Suarez, 2002). This environment impedes sperm progress and increases contact with the epithelium, contributing to the formation of the reservoir (Suarez, et al., 1997, Suarez, 2002). Sperm bind to the cilia or microvilli of oviduct epithelial cells via the plasma membrane over the acrosome (Lefebvre, et al., 1995, Suarez, 1998, Topfer-Petersen, 1999b, Suarez, 2002, Topfer-Petersen, et al., 2002). Sperm binding is mediated by carbohydrate recognition. The specific carbohydrate molecules involved vary among species (Lefebvre, et al., 1997, Topfer-Petersen, 1999a, Topfer-Petersen, 1999b, Green, et al., 2001, Suarez, 2002, Topfer-Petersen, et al., 2002, Wagner, et al., 2002). In cattle, sperm binding involves fucose recognition (Lefebvre, et al., 1997). The binding of sperm to oviductal cells involves a fucose containing ligand on the oviductal epithelium (Lefebvre, et al., 1997) and a calcium dependent lectin on the surface of bull sperm (Figure 1.1; Suarez, et al., 1998). Specifically, the protein on the sperm plasma membrane responsible for binding to the oviductal epithelium is Bovine Seminal Plasma Protein A1/A2 (BSP A1/A2), also called PDC-109 (Ignotz, et al.,

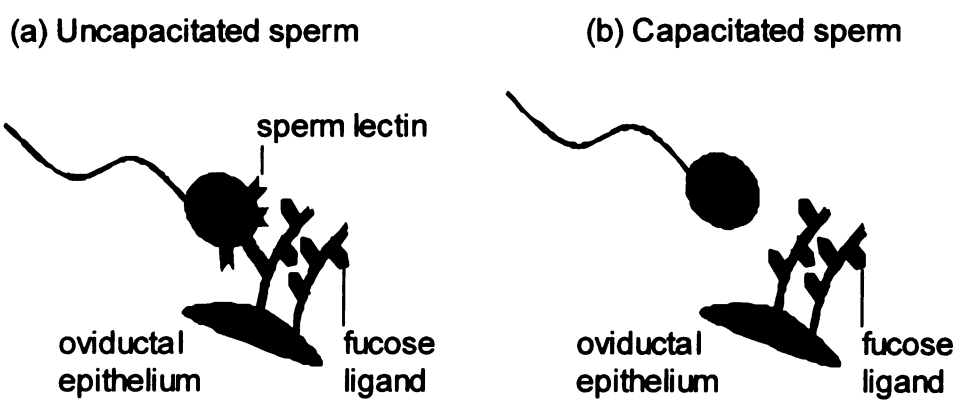


Figure 1.1: Sperm binding to (a) and release from (b) epithelium in the oviductal sperm reservoir. (a) Uncapacitated sperm bind to a fucose ligand on the oviductal epithelium via a lectin. (b) The lectin is lost or modified during capacitation, causing sperm to lose binding affinity for the epithelium and release from the reservoir. Figure modified from Suarez 1998.

2001, Gwathmey, et al., 2003). BSP A1/A2 associates with the sperm plasma membrane upon ejaculation and enables sperm to bind to oviductal cells (Gwathmey, et al., 2003).

One function of the oviductal sperm reservoir is to prevent polyspermic fertilization (Suarez, 1998, Suarez, 2002). The oviductal sperm reservoir may prevent polyspermy by allowing only a few sperm at a time to reach the oocyte (Day and Polge, 1968, Polge, et al., 1970, Hunter and Leglise, 1971, Hunter, 1972, Hunter, 1973). Polyspermy increased when sperm numbers were increased artificially in the pig oviduct by bypassing the sperm reservoir using various methods (Day and Polge, 1968, Polge, et al., 1970, Hunter and Leglise,

1971, Hunter, 1972, Hunter, 1973). Injection of progesterone prior to ovulation increased polyspermy (Day and Polge, 1968, Hunter, 1972). It was suggested that the effect of progesterone on polyspermy may have been due to an alteration of the environment of the oviduct, specifically, relaxation of the edematous tissues of the isthmus could have allowed increased numbers of spermatozoa to pass through the isthmus and reach the oocytes (Day and Polge, 1968, Hunter, 1972). Direct insemination of sperm in the oviducts also increased the incidence of polyspermy (Polge, et al., 1970, Hunter, 1973). These results indicated the functional importance of the lower isthmus in restricting the numbers of spermatozoa that reach the oocytes (Hunter, 1973). Finally, the surgical removal of the isthmus prior to insemination increased polyspermy, indicating that the isthmus normally limits the passage of spermatozoa to the upper regions of the oviduct (Hunter and Leglise, 1971).

Another important function of the oviductal sperm reservoir is maintenance of sperm fertility (Suarez, 1998, Topfer-Petersen, 1999b, Suarez, 2002, Topfer-Petersen, et al., 2002). Depending on the species, sperm may arrive in the reproductive tract of the female hours or days before ovulation (Topfer-Petersen, et al., 2002). During this time sperm motility and viability is maintained by binding to the oviductal epithelium in the sperm reservoir (Smith and Yanagimachi, 1990, Pollard, et al., 1991, Chian and Sirard, 1995, Smith and Nothnick, 1997). The ability of oviductal cells to maintain the fertilizing ability of sperm was demonstrated *in vitro* (Pollard, et al., 1991). Only sperm that were incubated with oviductal cells retained a capacity for fertilization when incubation lasted for 30 h

before *in vitro* fertilization (IVF). Tracheal epithelium or medium alone did not preserve sperm fertility (Pollard, et al., 1991).

During the pre-ovulatory period it is beneficial for sperm to remain bound to the epithelium in the oviductal sperm reservoir (Smith and Yanagimachi, 1990, Pollard, et al., 1991, Chian and Sirard, 1995, Smith and Nothnick, 1997, Suarez, 1998, Topfer-Petersen, 1999b, Suarez, 2002, Topfer-Petersen, et al., 2002, Hunter, 2003). However, sperm cannot fertilize an oocyte while bound to the oviductal epithelium (Suarez, 2002). Not only must sperm be released from the oviduct, sperm must be in the proper physiological state to fertilize the oocyte when ovulation occurs (Topfer-Petersen, 1999b, Suarez, 2002, Topfer-Petersen, et al., 2002). The physiological state of sperm is regulated in the oviductal reservoir to ensure that sperm capable of fertilization are available when the oocyte is receptive to fertilization (Chian and Sirard, 1995, Dobrinski, et al., 1997, Suarez, 1998, Fazeli, et al., 1999, Topfer-Petersen, 1999b, Suarez, 2002, Topfer-Petersen, et al., 2002, Hunter, 2003).

In addition to storage and regulation of the physiological state of sperm, the oviductal sperm reservoir selects viable and uncapacitated sperm for storage (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Topfer-Petersen, et al., 2002, Gualtieri and Talevi, 2003). The only sperm shown to be able to attach to oviductal epithelium were uncapacitated, acrosome intact, and morphologically normal (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Topfer-Petersen, et al., 2002). Further, fertilization competence was greater in sperm bound to oviductal epithelium, compared to unbound sperm and a control population of

sperm that had not been exposed to oviductal epithelium (Gualtieri and Talevi, 2003). Taken together, these results suggest that oviductal epithelium is able to select highly fertilization-competent sperm for storage, increasing the chances of a successful fertilization (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Topfer-Petersen, et al., 2002, Gualtieri and Talevi, 2003).

Binding to epithelium in the oviductal sperm reservoir maintains sperm viability during the preovulatory period (Pollard, et al., 1991, Smith and Nothnick, 1997, Suarez, et al., 1997, Suarez, 1998, Topfer-Petersen, 1999b, Scott, 2000, Suarez, 2002, Topfer-Petersen, et al., 2002). Release of sperm from the reservoir is necessary for fertilization to occur (Talevi and Gualtieri, 2001, Suarez, 2002). Also, sperm must be in the proper physiological state to fertilize oocytes (Topfer-Petersen, 1999a, Suarez, 2002). The binding affinity of oviductal cells for sperm does not change throughout the estrous cycle of the cow (Lefebvre, et al., 1995, Suarez, 1998, Suarez, 2002). Instead, changes in the sperm plasma membrane cause the loss of binding affinity for the oviductal cells (Figure 1.1; Lefebvre and Suarez, 1996, Suarez, 1998, Topfer-Petersen, 1999b, Revah, et al., 2000, Suarez, 2002, Gualtieri, et al., 2005).

Capacitation consists of a set of changes in the plasma membrane of sperm that enable the sperm to undergo the acrosome reaction following interaction with the zona pellucida, the oocyte's extracellular matrix (Therien, et al., 1995, Lefebvre and Suarez, 1996, Lane, et al., 1999, Therien, et al., 2001). The acrosome reaction is required for fertilization (Topfer-Petersen, 1999b, Topfer-Petersen, et al., 2002, Talbot, et al., 2003). The changes associated with

capacitation include loss of absorbed seminal plasma components from the sperm surface, modification of membrane lipid components, an increase in permeability to ions, an increase in intracellular calcium, redistribution of intramembranous and surface components, a change in internal pH, an increase in plasma membrane fluidity, a change in metabolism, and an increase in protein tyrosine phosphorylation (Therien, et al., 1995, Lefebvre and Suarez, 1996, Therien, et al., 1997, Therien, et al., 1998, Lane, et al., 1999, Therien, et al., 1999, Therien, et al., 2001). Capacitation can be triggered by high density lipoprotein (HDL) or heparin (Therien, et al., 1995, Therien, et al., 1997, Therien, et al., 1998, Lane, et al., 1999, Therien, et al., 2001, Gualtieri, et al., 2005). HDL and heparin trigger capacitation by different mechanisms (Lane, et al., 1999). Around the time of final sperm capacitation sperm also become hyperactivated (Ho and Suarez, 2001, Suarez and Ho, 2003). Hyperactivation is a movement pattern seen in sperm at the site and time of fertilization characterized by an increase in the flagellar bend amplitude and beat asymmetry (Ho and Suarez, 2001, Suarez and Ho, 2003). Hyperactivation enhances the ability of sperm to move around the oviduct lumen, penetrate mucus, and to penetrate the zona pellucida (Ho and Suarez, 2001, Suarez and Ho, 2003). Although hyperactivation alone cannot cause sperm to detach from the oviduct, it does enhance detachment by providing a force that helps the sperm pull away from the oviductal cell wall (Gualtieri, et al., 2005). Though both hyperactivation and capacitation occur around the same time, they are regulated by different pathways (Ho and Suarez, 2001, Suarez and Ho, 2003).

The membrane changes of capacitation may cause sperm release from the reservoir by modification or removal of binding components on the sperm surface (Figure 1.1; Talevi and Gualteri, 2001, Therien, et al., 2001). Agents used to capacitate sperm *in vitro* also cause sperm to be released from cultured oviductal cells (Talevi and Gualtieri, 2001). The BSP proteins that bind sperm to oviductal cells are released from the sperm membrane during capacitation (Therien, et al., 2001). These results indicate capacitation of sperm causes them to be released from the reservoir. Sperm release from the oviductal sperm reservoir must be coordinated with ovulation for fertilization to occur (Topfer-Petersen, 1999a, Topfer-Petersen, 1999b, Scott, 2000, Suarez, 2002, Topfer-Petersen, et al., 2002).

It has been shown that the hormonal state of the female does not control sperm binding by regulating the availability of binding sites on the oviductal cell surface (Lefebvre, et al., 1995, Suarez, 1998, Suarez, 2002). However, sperm release could be coordinated by regulating capacitation (Parrish, et al., 1989a, King, et al., 1994, Grippo, et al., 1995, Suarez, 1998, Suarez, 2002, Killian, 2004). Oviductal fluid is bio-chemically complex and contains many proteins including HDL and a heparin-like sulfated glycosaminoglycan (Parrish, et al., 1989b, Therien, et al., 1998, Lane, et al., 1999, Killian, 2004). Oviductal HDL levels are elevated during estrus (Therien, et al., 1998). Oviductal fluid collected during estrus stimulated sperm capacitation (Parrish, et al., 1989b, Grippo, et al., 1995). Thus, the oviduct is capable of regulating sperm binding by secreting capacitating agents prior to ovulation (Parrish, et al., 1989b, Grippo, et al., 1995,

Therien, et al., 1998, Lane, et al., 1999, Killian, 2004).

B. Summary of Life and Death of Sperm in the Female Reproductive Tract

After insemination, sperm travel through the female reproductive tract to the caudal isthmus of the oviduct (Hawk, 1983, Hunter and Wilmut, 1983, Scott, 2000, Hunter, 2003). Once in the oviduct, sperm form a reservoir by binding to the oviductal epithelium (Hunter and Wilmut, 1983, Scott, 2000, Hunter, 2003). Binding to the oviductal epithelium maintains sperm fertility (Smith and Yanagimachi, 1990, Pollard, et al., 1991, Smith and Nothnick, 1997). Capacitation causes sperm to be released from the reservoir and allows sperm to fertilize an oocyte (Talevi and Gualtieri, 2001, Therien, et al., 2001). Capacitation also decreases the life span of the sperm (Soupart and Orgebin-Crist, 1966, Bedford, 1970). Increasing the time from insemination to ovulation increases the amount of time sperm spend in the oviductal reservoir. Sperm that are released from the reservoir too early will no longer be viable when ovulation occurs. We hypothesize that X sperm may remain bound to the oviductal epithelium longer than Y sperm. The ability to remain attached to oviductal epithelium would allow the X sperm to retain the ability to fertilize oocytes for a greater length of time. Thus, a longer interval from insemination to ovulation would result in more X sperm being available to fertilize the oocyte and more females would be produced. The ability of X and Y sperm to remain attached to oviductal cells over time *in vitro* is discussed in Chapter Three.

III. DIFFERENCES BETWEEN X- AND Y-CHROMOSOME BEARING SPERM

X and Y sperm are produced through the process of spermatogenesis (Senger, 1999). Spermatogenesis consists of spermatocytogenesis, meiosis, and spermiogenesis (Senger, 1999). Spermatocytogenesis generates spermatogonia, the primitive diploid cells that will become the more advanced cell types (Senger, 1999). Stem cells divide mitotically to provide a continual source of spermatogonia (Senger, 1999). The mitotic divisions of the most advanced type of spermatogonia (B-spermatogonia) result in the formation of primary spermatocytes (Senger, 1999). One primary spermatocyte yields four spermatids through two meiotic divisions (Figure 1.2; Hendriksen, 1999, Senger, 1999). Meiosis begins when each chromosome is copied into two sister chromatids (Hendriksen, 1999). The homologous chromosomes separate during the first meiotic division and the sister chromatids separate during the second meiotic division (Hendriksen, 1999) The haploid spermatids differentiate into spermatozoa without dividing further through the process of spermiogenesis (Hendriksen, 1999, Senger, 1999). Each bovine spermatozoon contains 29 autosomes and either an X or a Y sex chromosome (Seidel and Garner, 2002).

The fundamental difference between sperm is the presence of either an X or a Y chromosome (Painter, 1924). Bovine X sperm contain approximately 3.8% more DNA than Y sperm due to the larger size of the X chromosome (Seidel and Garner, 2002). The X chromosome contains over ten times more genes than the Y chromosome (Alberts, et al., 2002).

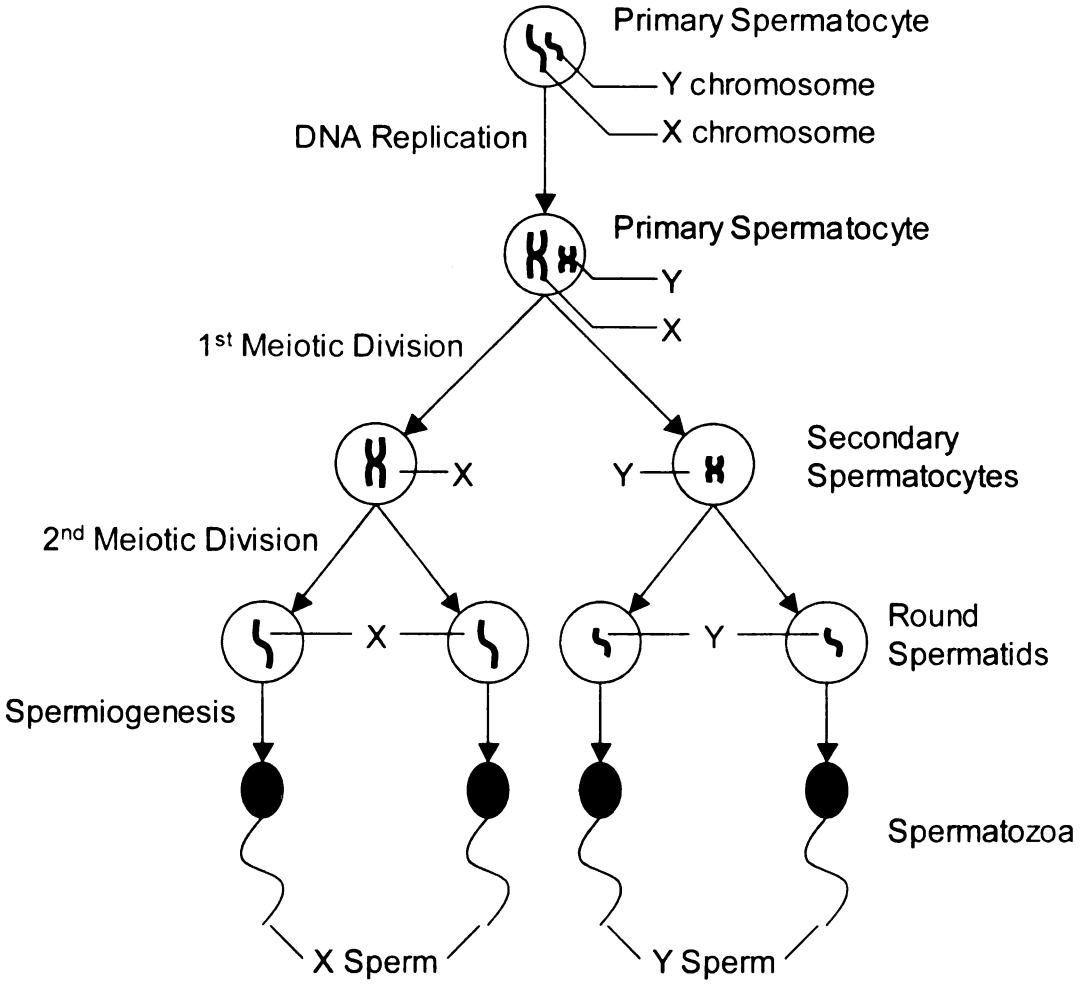


Figure 1.2: Meiosis and spermiogenesis. The first step of meiosis is replication of each chromosome of the primary spermatocyte into sister chromatids. The homologous chromosomes separate during the first meiotic division, creating two secondary spermatocytes. The sister chromatids separate during the second meiotic division, creating four round spermatids with a haploid amount of DNA. The haploid spermatids differentiate into spermatozoa without dividing further through spermiogenesis. One primary spermatocyte yields two X sperm and two Y sperm.

Genetic differences can translate to phenotypic differences. Many researchers have searched for phenotypic differences between X and Y sperm. Phenotypic differences that have been studied in X and Y sperm include size, density, surface molecules, motility, and survival rates. Despite the differences between the sex chromosomes, few phenotypic differences between X and Y sperm have been identified (Seidel and Garner, 2002).

A. Separation of X Versus Y Sperm Based on Structural Differences

Because the X chromosome is larger than the Y chromosome, X sperm contain more DNA than Y sperm (Seidel and Garner, 2002). Differences in size and density have been suspected to exist between X and Y sperm because X sperm must accommodate more DNA than Y sperm (van Munster, et al., 1999, Hossain, et al., 2001, Seidel and Garner, 2002, Revay, et al., 2004). Studies to determine size differences between X and Y sperm yielded contradictory results (Cui, 1997, van Munster, et al., 1999, Hossain, et al., 2001, Revay, et al., 2004). One study found a difference in the head volume of flow sorted bovine sperm (van Munster, et al., 1999). This difference was equivalent to the difference in DNA content between X and Y sperm, 3.5 to 4 percent (van Munster, et al., 1999). However, no statistical analysis was given to indicate is these differences were statistically significant (van Munster, et al., 1999). Another study found no differences between live bovine X and Y sperm in head area (Revay, et al., 2004). In this study head area was measured after identifying morphologically normal, live sperm (Revay, et al., 2004). X and Y sperm were determined using a

previously validated FISH method (Rens, et al., 2001, Revay, et al., 2004). The contradictory results of these two studies may be due to the different methods used. Processing and staining procedures were found to influence the size measurements of sperm heads (Foote, 2003). A study on human sperm compared X and Y sperm head area, length, and width and tail length after using different treatments prior to hybridization with FISH probes (Hossain, et al., 2001). This study found that with identical treatment, X and Y sperm did not have significant morphological differences (Hossain, et al., 2001). The authors felt these findings were not an artifact of the methodology because "it is highly unlikely that all prehybridization treatments utilized in this study would work to the same extent in the same direction to eliminate the pre-existing differences of X and Y, if any existed" (Hossain, et al., 2001). It is not clear whether bovine X and Y sperm differ in size at this time. However, if differences do exist they would be slight and therefore it is unlikely that procedures to separate sperm based on differences in size would be sensitive enough to distinguish between sperm of the two populations.

The difference in the amount of DNA in X and Y sperm could cause X sperm to have a slightly higher density (van Munster, et al., 1999). Separation of human sperm on discontinuous density gradients produced separated fractions that were enriched for X sperm (Andersen and Byskov, 1997, Lin, et al., 1998). One technique increased the percent X sperm from 51.8 percent in controls to 60.7 percent, other techniques used gave less enrichment (Andersen and Byskov, 1997, Lin, et al., 1998). Discontinuous density gradients used to

separate sperm into subpopulations were unable to separate bovine sperm into subpopulations containing different ratios of X and Y sperm (Luderer, et al., 1982, Upreti, et al., 1988). While differences in density may exist between X and Y bovine sperm, such differences were not large enough to allow for their separation (Upreti, et al., 1988). Random variation in sperm cell density appears to overshadow differences due to the X and Y chromosomes (Luderer, et al., 1982).

The different genes present on the X and Y chromosomes may result in expression of different molecules on X versus Y sperm (Dym and Fawcett, 1971, Morales and Hecht, 1994, Hendriksen, 1999, Alberts, et al., 2002). The following studies investigated possible differences between X and Y sperm in surface membrane proteins. No differences were detected between the surfaces of X and Y sperm (Hafs and Boyd, 1974, Ali, et al., 1990, Cartwright, et al., 1991, Cartwright, et al., 1993, Hendriksen, et al., 1993, Hendriksen, et al., 1996, Howes, et al., 1997, Checa, et al., 2002).

Differences in surface molecules could cause X and Y sperm to have different surface charges. Little differences were found in sperm using charge-sensitive aqueous two-phase partition, a precise system that was used to partition cells based on different surface molecules (Cartwright, et al., 1991). Although differences between X and Y sperm were not studied directly, these results showed that bovine sperm did not have detectable surface charge heterogeneity (Cartwright, et al., 1991). When electrophoresis was used to separate sperm on the basis of charge no differences were found in the ratio of X

and Y sperm (Checa, et al., 2002) or the sex of calves born (Hafs and Boyd, 1974). Taken together, these studies indicate that X and Y sperm do not differ in surface charge (Hafs and Boyd, 1974, Cartwright, et al., 1991, Checa, et al., 2002). While this is in opposition to early studies claiming differences in charge between X and Y sperm (Kaneko, et al., 1983, Kaneko, et al., 1984, Engelmann, et al., 1988, Ishijima, et al., 1991, Blottner, et al., 1994), the earlier studies were later invalidated because an inaccurate method was used to distinguish X and Y sperm (Vankooij and Vanoost, 1992).

Although no differences were found based on charge, two populations of sperm having different surface properties were found using charge-insensitive aqueous two-phase partition (Cartwright, et al., 1991). These two populations showed heterogeneity of non-charged surface molecules and it was thought that these two populations represented X and Y sperm (Cartwright, et al., 1991). However, later studies using the same system found that the populations separated contained both X and Y sperm (Cartwright, et al., 1993). It was concluded that the separation was not based on differences in surface molecules between X and Y sperm (Cartwright, et al., 1993). The separation was based, at least in part, on whether the sperm were acrosome reacted (Cartwright, et al., 1993).

The work discussed above utilized sperm populations that contained both X and Y sperm together. More recently, studies have compared X and Y sperm populations separated using flow cytometry (Hendriksen, et al., 1996, Howes, et al., 1997). No differences were found in protein spots between boar X and Y

sperm fractions using high resolution two-dimensional electrophoresis (Hendriksen, et al., 1996). High resolution two-dimensional electrophoresis was also used to compare X and Y chromosome enriched populations of bull sperm (Howes, et al., 1997). A small cluster of proteins was found to be unique to the X chromosome enriched population, however, it was determined that these were not surface membrane proteins (Howes, et al., 1997). No differences were found between the X and Y chromosome enriched populations using one-dimensional electrophoresis or production of monoclonal antibodies (Howes, et al., 1997). These results suggest that X and Y sperm are phenotypically identical (Hendriksen, et al., 1996). A difference was found between sorted and unsorted sperm using one-dimensional electrophoresis, indicating the sorting process modified the sperm surface membrane (Howes, et al., 1997). Differences in surface proteins may not have been detected because proteins were lost during the sorting process. Also, the techniques used may not have been able to detect differences in very small peptides such as the H-Y antigen (Hendriksen, 1999).

The H-Y antigen is a small, male specific antigenic epitope that was found on the surface of sperm (Hendriksen, 1999). Some early studies found that treating sperm with anti-H-Y antibodies prior to insemination slightly increased the number of females born (Bennett and Boyse, 1973, Zavos, 1983). Studies that looked directly for the H-Y antigen on sperm surfaces yielded mixed results (Goldberg, et al., 1971, Ali, et al., 1990, Hendriksen, et al., 1993). Differences in the H-Y antigen between X and Y sperm are unlikely because the genes encoding the H-Y epitopes have homologues on the X chromosome (Hendriksen,

1999).

The nature of spermatogenesis makes it unlikely that differences in the cell surface of X and Y sperm exist (Hendriksen, 1999). Cytokinesis is not complete during the mitotic and meiotic divisions of spermatogenesis (Dym and Fawcett, 1971, Hendriksen, 1999). Sperm cells remain connected by intercellular bridges. These intercellular bridges allow sharing of gene products between X and Y sperm (Braun, et al., 1989, Hendriksen, 1999). Gene transcription stops before the intracellular bridges are disrupted, making it unlikely that the X and Y sperm differences would occur as a result of gene transcription after the disruption of intracellular bridges (Dym and Fawcett, 1971, Morales and Hecht, 1994, Hendriksen, 1999). While it has been shown that some gene products move between the intercellular bridges that connect developing spermatids, not all transcripts and proteins have been tested (Braun, et al., 1989). It is possible that not all gene products flow freely through these bridges. Unequal gene product sharing could be a possible mechanism to create differences in the cell surfaces of X and Y sperm, though there is no direct evidence for this mechanism (Hendriksen, 1999).

B. Separation of X Versus Y Sperm Based on Functional Differences

Besides structural differences, X and Y sperm may also have functional differences (Shettles, 1970, Hossain, et al., 1998). Sperm motility was suspected to vary between X and Y sperm (Shettles, 1970, Ericsson, et al., 1973, Hossain, et al., 1998, Penfold, et al., 1998). Methods to select highly motile sperm include

Percoll density gradient centrifugation, swim-up, and albumin gradients (Han, et al., 1993a, Kobayashi, et al., 2004). The ability of these methods to separate X and Y sperm based on motility was studied extensively, with mixed results (Upreti, et al., 1988, Beernink, et al., 1993, Han, et al., 1993a, Pyrzak, 1994, Wang, et al., 1994, Aribarg, et al., 1996, Chen, et al., 1997, De Jonge, et al., 1997, Lin, et al., 1998, Madrid-Bury, et al., 2003, Kobayashi, et al., 2004). There is no conclusive evidence that either X or Y sperm swim faster than the other (Wang, et al., 1994, Penfold, et al., 1998). In stationary fluids, X and Y sperm swim at the same speed (Sarkar, et al., 1984, Penfold, et al., 1998) but differ in their linearity and straightness of path (Penfold, et al., 1998). However, *in vivo* sperm are not in stationary fluid, instead, they are in a complex flow environment (Sarkar, et al., 1984). When placed in a flow-stream, sperm shift to a straighter path of movement with a decreased angular velocity. This shift is four times more pronounced in X sperm (Sarkar, et al., 1984). These results indicate X and Y sperm differ slightly in some aspects of motility (Sarkar, et al., 1984, Penfold, et al., 1998). Subtle differences between the swimming speeds of X and Y sperm could also exist, yet remain undetected, because of limitations in the sensitivity of experimental procedures (Penfold, et al., 1998).

One functional difference found between X and Y sperm is their rates of survival (Van Dyk, et al., 2001, Lechniak, et al., 2003). Human Y sperm exhibited a loner functional survival under *in vitro* conditions compared to X sperm (Van Dyk, et al., 2001). More Y sperm were capable of binding to the zona pellucida than X sperm after 48 h of incubation (Van Dyk, et al., 2001). In cattle, the

opposite seems to be true. When sperm were pre-incubated for 24 h prior to *in vitro* fertilization, more female hatched blastocysts were produced compared with fresh sperm (Lechniak, et al., 2003). Dead sperm cannot attach to or remain attached to oviductal epithelium in the sperm reservoir (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Topfer-Petersen, et al., 2002). Thus, the longer survival of X sperm may also mean X sperm are able to remain attached to the oviductal epithelium longer than Y sperm.

C. Identification and Quantification of X- and Y-Chromosome Bearing Sperm

As indicated above, there are few identified phenotypic differences between X and Y sperm. Therefore, methods developed to identify and quantify X and Y sperm have focused on detecting the X or Y chromosome, DNA sequences found on the X or Y chromosome, or differences in total DNA content.

One of the first reported methods developed to identify Y sperm was a quinacrine dihydrochloride stain. This stain was thought to bind to the distal half of the long arm of the human Y chromosome, producing a florescent Y-body (Pearson and Bobrow, 1970). However, this procedure was later found to be inaccurate when compared with karyotyping (Ueda and Yanagimachi, 1987) or DNA probes (Vankooij and Vanoost, 1992). Not all Y sperm had a Y-body and not all sperm with a Y-body were Y sperm (Ueda and Yanagimachi, 1987).

A method to visualize the chromosome content of an individual sperm was developed (Rudak, et al., 1978). Karyotyping of sperm was accomplished

when sperm fused with zona-free hamster oocytes (Rudak, et al., 1978, Tateno and Mikamo, 1987, Ueda and Yanagimachi, 1987). After fusion, the sperm chromatin expanded and individual chromosomes were identified (Rudak, et al., 1978, Tateno and Mikamo, 1987, Ueda and Yanagimachi, 1987). This method was shown to be reliable and repeatable for identification of both human (Ueda and Yanagimachi, 1987) and bovine (Tateno and Mikamo, 1987) X and Y bearing sperm. However, this method is costly and time consuming because every sperm must be analyzed individually (Hassanane, et al., 1999, Piumi, et al., 2001).

DNA content of sperm nuclei were measured accurately using flow cytometry (Pinkel, et al., 1982, Garner, et al., 1983, Pinkel, et al., 1985, Johnson, et al., 1987a, Welch and Johnson, 1999). The flat shape and high refractive index of mammalian sperm nuclei make optical measurements of sperm DNA sensitive to sperm head orientation (Pinkel, et al., 1982). In order to measure sperm DNA content using flow cytometry, the instrument must be modified. Modifications needed are a special nozzle to orient the sperm head with the flat side facing the laser and a second fluorescence detector to determine if the sperm head is properly oriented (Johnson, et al., 1987b). Flow cytometric measurements of X and Y sperm DNA content were in agreement with length-based estimates (Pinkel, et al., 1985). Flow cytometry and FISH performed equally well in determining proportions of X and Y sperm in samples (Welch and Johnson, 1999). The best resolution of flow cytometric analysis of sperm were obtained after sonication to remove tails and staining with Hoechst (Johnson, et

al., 1987a).

DNA probes specific for the sex chromosomes made it possible to use southern blots for sexing sperm (Beckett, et al., 1989, Sarkar, 1989, Vankooij and Vanoost, 1992). In this method, DNA was digested and transferred to a membrane. DNA probes that recognize distinguishable loci on one or both of the sex chromosomes were visualized as autoradiographic bands (Beckett, et al., 1989, Sarkar, 1989, Vankooij and Vanoost, 1992). These bands were analyzed to determine relative DNA content and the proportion of X and Y sperm (Beckett, et al., 1989, Sarkar, 1989, Vankooij and Vanoost, 1992). DNA-DNA in situ hybridization was also used to determine the sex chromosome compliment of individual sperm (West, et al., 1989). In this method, DNA probes that identified the Y chromosome were hybridized to de-condensed sperm DNA. Brightfield microscopy was used to identify the hybridization spots and distinguish Y sperm. Sperm without spots were assumed to be X sperm (West, et al., 1989).

Another technique used to detect genes on the sex chromosome of spermatozoa is FISH. FISH requires a direct or indirect fluorescent DNA probe complimentary to a specific gene target sequence (Parrilla, et al., 2003). For identification of X and Y sperm, these probes were targeted to one or both sex chromosomes (Han, et al., 1993b, Hassanane, et al., 1999, Piumi, et al., 2001, Rens, et al., 2001, Parrilla, et al., 2003, Revay, et al., 2003, Di Berardino, et al., 2004). Because of the high degree of condensation of sperm DNA, sperm nuclei were de-condensed before hybridization, to allow the probes access to the hybridization sites (Piumi, et al., 2001, Parrilla, et al., 2003, Revay, et al., 2003,

Di Berardino, et al., 2004). After de-condensation and hybridization, sperm were identified as X or Y based on the color spot that appeared on each individual sperm, corresponding to the different probes. Double FISH using indirect probes for both X and Y was used to identify X and Y sperm in cattle (Hassanane, et al., 1999, Piumi, et al., 2001, Rens, et al., 2001), humans (Han, et al., 1993b), water buffalo (Revay, et al., 2003, Di Berardino, et al., 2004) and sheep and goats (Di Berardino, et al., 2004). In these studies, X sperm were identified as sperm having green spots and Y sperm as sperm having red spots.

Indirect probes were useful in detecting specific DNA sequences but they required at least one, and usually up to three, detection steps before visualization under a microscope (Parrilla, et al., 2003). These extra steps made the use of indirect probes slower than direct labeling (Parrilla, et al., 2003). Although direct FISH required longer DNA targets, it was faster than using indirect probes (Parrilla, et al., 2003). Nick-translated probes gave the highest sensitivity compared with probes labeled by other methods (Parrilla, et al., 2003). Direct DNA probes specific to the Y chromosome and chromosome one (used as an internal control) prepared using nick translation were successfully used to sex porcine sperm (Parrilla, et al., 2003).

PCR is another technique that can use DNA sequences of the X or Y chromosome to identify and quantify X and Y sperm. PCR is a technique to amplify a specific region of DNA using sequence-specific primers and multiple cycles of DNA synthesis (Mullis and Faloona, 1987). The basic PCR run has three phases, the exponential phase in the beginning when an exact doubling of

product is accumulated, the linear phase in the middle when the reaction is slowing as components are being consumed, and the plateau phase at the end, after the reaction has stopped (Valasek and Repa, 2005). In traditional PCR amplification products are measured after the reaction has stopped by gel or capillary electrophoresis (Ishijima, et al., 1992, Chandler, et al., 1998, Checa, et al., 2002). However, the amount of starting DNA cannot be reliably calculated by quantifying the amount of product at the completion of the PCR because the reaction is only able to amplify the DNA efficiently up to a certain quantity before the plateau effect occurs (Valasek and Repa, 2005). In contrast, real-time PCR measures product formation during the exponential phase, while the DNA is being amplified efficiently (Valasek and Repa, 2005). Measurement at this phase allows precise quantification of the starting DNA sequences of interest (Valasek and Repa, 2005).

Traditional PCR was first used to detect differences in Y chromosome content of samples by comparing the optical density of gel bands produced by the PCR products after electrophoresis (Ishijima, et al., 1992). Quantification of numbers or percent Y sperm was not possible (Ishijima, et al., 1992). Later, a traditional PCR method to calculate percent Y sperm in samples was developed using a pooled standard of DNA that was assumed to contain 50% Y sperm (Chandler, et al., 1998).The amplified DNA fragments were stained and electrophoresed on agarose gels (Chandler, et al., 1998). The intensity measures of gel bands for each sample were compared with that of the pooled standard to calculate the relative amount of Y chromosome DNA in each sample (Chandler,

et al., 1998).

Further improvements to traditional PCR sexing of sperm came in 2002 when a method to quantify the X and Y chromosomes present in samples was developed using DNA pooling, amplification of the amelogenin gene, and capillary electrophoresis (Checa, et al., 2002). DNA pools with different known amounts of X and Y chromosome content were prepared with bull and cow blood DNA. The DNA pools were used to estimate regression parameters and the corresponding regression function was used to predict the relative X chromosome frequency in samples (Checa, et al., 2002).

Real-time PCR was used to quantify the proportion of Y sperm in samples (Joerg, et al., 2004, Parati, et al., 2006). One method used the comparison of cycles to threshold (C_T) for the amplification of two different genes, a Y specific gene and an autosomal gene to determine the proportion of X and Y sperm (Joerg, et al., 2004). In a more recent report primers and probes were designed for the X and Y chromosomes and plasmid cDNA clones were used as a standard reference (Parati, et al., 2006). The Y specific primers were designed on a conserved region of the bovine Y-chromosome-linked SRY gene and the X-specific primers were designed on the intron 2 region of the bovine proteolipid protein gene (Parati, et al., 2006). This new method was shown to be reliable for quantifying the sex chromosome content of sperm samples.

D. Summary of Differences Between X- and Y-Chromosome Bearing Sperm

Spermatozoa are responsible for determining offspring sex (Painter, 1923,

Painter, 1924). X sperm produce females and Y sperm produce males (Painter, 1923, Painter, 1924). Desire to control offspring sex has led to interest in finding differences between X and Y sperm and developing methods to identify and quantify X and Y sperm.

Few significant differences between X and Y sperm have been found despite the numerous studies conducted on the subject. Some aspects that have been shown to have little or no differences between X and Y sperm are sperm size, density, surface molecules, and motility. One difference found between X and Y sperm was the survival rate. In cattle, X sperm survived longer than Y sperm *in vitro* (Lechniak, et al., 2003). The underlying mechanism for the apparent difference in survival between X and Y sperm in unclear (Lechniak, et al., 2003). The main difference between X and Y sperm is the presence of the X or Y chromosome (Painter, 1924).

The X and Y chromosomes differ greatly in gene content (Alberts, et al., 2002). Thus, techniques developed to identify and quantify X and Y sperm have been based on differences in the X and Ychromosomes. Techniques developed thus far are limited by low accuracy, low throughput, high cost, and technical difficulty. A new technique to quantify Y sperm is presented in Chapter Two of this thesis.

IV. TIMING OF INSEMINATION IN RELATION TO OVULATION ALTERS GENDER RATIO

Ovulation synchronization techniques allow inseminations to be accurately

timed to subsequent ovulations. The Ovsynch protocol synchronized the time of ovulation within an 8 h period (Pursley, et al., 1995). Ovulation occurred 24 to 32 h after the last injection of Ovsynch (Pursley, et al., 1995). The precise synchrony of ovulation allows for an effective test to determine the effect of the time of insemination relative to ovulation on sex ratio (Pursley, et al., 1998). Cows were inseminated at different times in relation to the last injection of Ovsynch (Pursley, et al., 1998). These times corresponded to 24 to 32 h before ovulation, 16 to 24 h before ovulation, 8 to 16 h before ovulation, and 0 to 8 h before ovulation (Pursley, et al., 1998). Significantly more female calves (61%) were produced when cows were inseminated 24 to 32 h before ovulation (Pursley, et al., 1998).

These results were confirmed later in a study that compared inseminations performed either 32 to 40 h or 8 to 16 h before ovulation in a large number of cows (Macfarlane, 2003). In this study Ovsynch was again used to synchronize ovulation (Macfarlane, 2003). Early insemination resulted in 65% females compared to 56% females in the other group (Macfarlane, 2003).

Possible mechanisms that may be responsible for the effect of time of insemination on sex ratio include differential transport of sperm through the female tract, differences in capacitation times or functional survival between X and Y sperm, preferential selection of sperm, or sex related embryo death. In humans, more Y sperm remained functional after extended *in vitro* incubation at 37°C (Van Dyk, et al., 2001). Duration of capacitation has an effect on the prevalence of X and Y sperm (Barczyk, 2001). Longer capacitation times favored Y sperm while shorter times favored X sperm in humans (Barczyk, 2001). Taken

together, these findings indicate that human Y sperm remain viable longer than human X sperm and that this may be due to different rates of capacitation between X and Y sperm (Barczyk, 2001, Van Dyk, et al., 2001). Extended *in vitro* incubation of bull sperm produced more female hatched blastocysts (Lechniak, et al., 2003). This result indicates that in cattle, X sperm have longer functional survival or delayed capacitation compared to the Y sperm (Lechniak, et al., 2003).

Other *in vitro* studies indirectly support the idea that X and Y sperm have different functional survival or rates of capacitation. When bovine oocytes were inseminated immediately after maturation, more females were detected, in contrast, when insemination was delayed more males were produced (Dominko and First, 1997, Gutierrez-Adan, et al., 1999). It was suggested that these differences may have been due to the oocyte having differing ability to process X and Y sperm depending on its maturational status (Dominko and First, 1997). However a recent study showed that oocytes are not selective towards X or Y sperm (Zuccotti, et al., 2005). Another possible explanation is that Y sperm respond earlier and reach fertilizing ability first (Gutierrez-Adan, et al., 1999), so when IVF is delayed Y sperm are favored, but when IVF is immediate the oocyte is not yet capable of being fertilized so the early response of Y sperm leads to its loss of fertilizing ability before the oocyte becomes receptive, leaving the slower-responding X sperm at an advantage. In another study, a short sperm-oocyte co-incubation time during IVF produced more males, while extending the co-incubation time caused the sex ratio to equalize (Kochhar, et al., 2003). This

supports the idea that the Y sperm have an advantage in fertilizing ability early and lose this ability over time, when the X sperm gain the advantage.

The overall hypothesis of the current thesis research is that X sperm are able to remain attached to oviductal epithelium longer than Y sperm. The following objectives were put forth to test this hypothesis:

OBJECTIVE 1: Develop a quantitative real-time based PCR assay to determine the proportion of Y sperm in sperm samples from cattle;

OBJECTIVE 2: Determine if the proportion of X and Y sperm attached to *in vitro* cultured oviductal epithelial cells changes over time.

The research presented in Chapter Two of this thesis is novel because it provides a reliable, high throughput method to quantify Y sperm in samples from cattle. This method will be useful for determining differences between X and Y sperm as well as for validating methods to separate X and Y sperm for production of offspring. The research presented in Chapter Three of this thesis showed for the first time that more Y sperm were released from oviductal cells during co-incubation than X sperm.

CHAPTER TWO

A Real-Time Polymerase Chain Reaction Assay for Quantification of Y-Chromosome Bearing Sperm in Cattle

I. ABSTRACT

Recent data from our laboratory indicates that increasing the time from AI until ovulation in cattle affects sex ratio of offspring in favor of females (Pursley, et al., 1998, Macfarlane, 2003). One possible explanation for this phenomenon is that the population of X and Y sperm deviate over time at the detriment of Y sperm. Thus, at the time of ovulation, fewer Y sperm would constitute the population available for fertilization. *In vitro* co-culture of sperm and oviductal cells is one way to test if a greater percentage of Y sperm detaches from oviductal cells following prolonged incubation. In order to test this, a reliable, high throughput method to determine numbers of Y sperm is required. The objective of this study was to develop and validate a quantitative PCR based assay to accurately determine numbers of Y sperm in samples from cattle. Primers were designed to amplify a 21 base pair (bp) section of autosomal DNA from the 1.715 satellite (for estimation of total sperm numbers) and a 57 bp section of the SRY gene (for quantification of Y sperm). Standard curves were developed from plasmids containing cloned fragments of the SRY gene and the 1.715 satellite region from which primers were designed. Average R^2 of 0.9977 and 0.9886 were obtained for the 1.715 satellite and SRY standard curves. Assay of fixed amounts of male genomic DNA revealed between and within assay CV of 2.17%

and 1.54% for the 1.715 satellite and 8.79% and 9.08% for the SRY assay, respectively. Specificity of the assay for the SRY gene was verified using female and male genomic DNA. Similar copy numbers per unit DNA were obtained for the 1.715 satellite sequence in both male and female DNA samples, but the SRY sequence was undetectable in female DNA samples. A method to remove somatic cell DNA from sperm samples before extraction of Sperm DNA was validated. Samples of separated semen with known amounts of X and Y sperm (donated by XY Inc.) were used to determine accuracy of the real-time PCR assay in quantification of numbers of Y sperm. DNA was extracted from sorted semen samples containing 16.8%, 32.0%, 66.5%, and 79.0% Y sperm and copies of SRY and 1.715 satellite DNA determined in duplicate samples. Percent recovery of genomic DNA from sorted semen samples was calculated using 1.715 satellite primers and used to adjust expected number of SRY copies for each sample of sorted semen analyzed. Differences in observed copies of SRY gene versus expected copies of SRY gene were determined by Student's t test. No differences in observed versus expected copies of the SRY gene were detected for each sample ($P > 0.05$). In conclusion, an assay that accurately quantifies numbers of Y sperm was developed and validated. This assay was used in Chapter Three of this thesis to determine if the proportion of Y sperm that remained attached to oviductal cells changed over time.

II. INTRODUCTION

A reliable, high throughput method for determining the proportions of X

and Y sperm could be a useful tool for finding the answers to basic biological questions about potential differences between X and Y sperm in their behavior or survival in the female reproductive tract under various conditions. Information on this subject could help elucidate mechanisms that affect gender ratio of offspring. Such a tool would also be useful for validating methods of sperm manipulation for predetermining the sex of offspring.

Our laboratory is interested in effects of time of insemination relative to ovulation on gender ratio of offspring. We have found that increasing the time from insemination to ovulation affects sex ratio of offspring in favor of females (Pursley, et al., 1998, Macfarlane, 2003). One possible explanation for this is that X and Y sperm populations deviate over time at the detriment of Y sperm. A reliable, high-throughput method for quantification of numbers of Y sperm is needed for testing of the above hypothesis (Chapter Three). Quantitative real-time PCR represents a potential reliable, high-throughput method for quantification of numbers of Y sperm. The objective of this study was to develop and validate a quantitative real-time PCR based assay that accurately quantifies numbers of Y sperm.

III. MATERIALS AND METHODS

A. Standard Curves

A section of the bovine 1.715 satellite and a portion of the SRY gene were amplified by traditional PCR using two sets of primers (Table 2.1). The primers used to amplify the 1.715 satellite were published (Machaty, et al., 1993) and the

Table 2.1: Primer Sequences			
Sequence	**Direction**	**PCR Primers**	**Real-Time PCR Primers**
1.715 Satellite	Forward	CCCTTCCAGCTGCAGTGTCA	GATTGGTCTCTAGGCCATGCA
	Reverse	GATCTGTAACTGCAAACCTGGC	GAGATTCCCCCGTCATCGA
SRY Gene	Forward	GAACGCTTACACCGCATATT	GCTATGTTCAGAGTATTGAACGACG
	Reverse	TAACATGTTCTCCCCTTTCA	GCGCTATGACTGTCTGTGCAC

primers used to amplify the SRY gene were designed based on the sequence of a portion of the gene (Joerg, et al., 2004). The PCR components were 0.25 μl of Taq DNA Polymerase, 2μl 10x buffer, 0.5 μl $MgCl_2$ (Invitrogen Corporation; Carlsbad, CA), 1μl each forward and reverse primer (5μM), 0.4 μl 10mM dNTP, and 2 μl of 95.2 ng/μl bovine genomic DNA, adjusted with Nuclease Free Water (Ambion; Austin, TX) to 21 μl. The PCR conditions were an initial 5 min at 94°C,

(a) 1.715 Satellite

TGGAAGCAAA GAACCCCGCT CTGCTCTCGA ATCGCGACGG GTATCTCTTG GAGCTCACTG GGTGGACTCA AGGGAGTCAA GCCTCCTGAG GCGTTTGGAG AGAGGTCGCG **AGATTGGTCT CTAGGCCATG CA**GGAGACGA AGGCCCTCAT CTC**TCGATGA CGGGGGAATC TC**GGGGTTGT TCTCGAGCGG CGGCCCCAGT GTGCGGTTTC TCACGA

(b) SRY Gene

GAACGCTTAC ACCGCATATT ACTTCCTCCC CTTTTAAACA GTGCAGTCGT ATGCTTCT**GC TATGTTCAGA GTATTGAACG ACG**ATGTTTA CAGTCCAGCT GTGGTACAGC AACAAACTAC TCTCGCTTTT AGGAAAGACT CTTCCTT**GTG CACAGACAGT CATAGCGC**AA ATGATCAGTG TGAAAGGGGA GAACATGTTA

Figure 2.1: Sequence of amplified sections of the bovine (a) 1.715 satellite and the (b) SRY gene; real-time PCR primer annealing sites denoted in bold.

35 cycles of 95°C for 60 s, 59°C for 60 s, 72°C for 120 s or 90 s (for the 1.715 satellite and the SRY gene respectively), and a final 10 min at 72°C. 4 μl of the resulting amplified product was ligated into a pCR2.1-TOPO vector (Invitrogen Corporation; Carlsbad, CA). The ligation was transformed into chemically competent *E. coli,* grown overnight on Agar plates containing 50 μg/ml ampicillin, 100 μl 100mM IPTG and 40 μl of 40mg/ml Xgal. Colonies containing the PCR product insert (identified by their white color) were selected and grown overnight in LB medium or Terrific Broth (for the 1.715 satellite and the SRY gene respectively) containing 50 μg/ml ampicillin. The DNA was purified from the E. coli using the Wizard® PlusSV Minipreps DNA Purification System (Promega; Madison, WI). DNA was sequenced at the Michigan State University Genomics Technology Support Facility. The sequences appear in Figure 2.1. Standard dilutions of the plasmids were produced by serial dilution in Nuclease Free Water. Average R^2 was attained for the standard curves of each plasmid set using two real-time PCR runs.

B. Somatic Cell DNA Extraction

Somatic cell DNA was extracted from male (liver) and female (corpus luteum) tissue samples. Frozen tissue was placed in liquid nitrogen and pulverized. 30 ml digestion buffer (100mM NaCl, 10mM TrisCL, 25mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K, pH 8) was added to the tissue powder and the mixture shaken at 50°C for 12h. DNA was recovered using P;C:I extraction (25:24:1; Sigma Chemical Company; St. Louis, MO) described below.

C. Somatic Cell DNA Removal and Sperm DNA Extraction

A previously published method was used to extract sperm DNA (Gill, et al., 1985). Samples were centrifuged at 15000 x g for 5 min and the supernatant was removed. An Epithelial Lysis Mixture (600 μl; 0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl, 2% SDS, 20 μg/ml proteinase K) was added to each sample to lyse any non-sperm cells while leaving sperm nuclei intact. Samples were incubated at 37°C for 30 min. Sperm nuclei were centrifuged at 15000 x g for 5 min, washed with 1 ml of 70% ethanol, and centrifuged again at 15000 x g for 5 min. The supernatant was removed by pipetting after each centrifugation. Sperm were incubated for 12-14 h at 37°C in 200 μl of a Sperm Lysis Mixture (0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl, 2% SDS, 0.039 M DTT, 20 μg/ml proteinase K). DNA was recovered using P:C:I extraction.

P:C:I extraction was performed to recover DNA from male and female tissues and sperm after digestion. An equal volume of P:C:I was added to the digested tissue in a conical tube and vortexed. The mixture was centrifuged for 10 min at 1700 x g. The supernatant was placed in a new conical tube and the P:C:I extraction repeated once. 3.5 ml of 7.5M ammonium acetate and 14 ml of 100% ethanol were added to the supernatant. The samples were centrifuged for 10 min at 2000 x g and the supernatant was discarded. The samples were washed in 1 ml of 70% ethanol and centrifuged for 5 min at 15000 x g. The supernatant was discarded and the tubes were air dried to remove remaining ethanol. The pellet was suspended in 500 μl TE buffer for the male and female tissue DNA or in 50 μl Nuclease Free Water for the sperm DNA.

D. Real-Time PCR

Real-time PCR primers were designed to amplify a 21 bp section of autosomal DNA from the 1.715 satellite using the Primer Express program (Applied Biosystems; Foster City, CA). Real-time PCR primers published previously were used for amplification of a 57 bp section of the SRY gene (Joerg, et al., 2004). Primer sequences are listed in Table 2.1. The 1.715 primers were used to determine the total number of sperm and the SRY primers were used to determine the number of Y sperm for an assay to determine the number of Y sperm in cattle. The assay was validated using DNA from male liver cells, female corpus luteum cells, and sperm.

Real-time PCR was performed by ABI PRISM 7000 Sequence Detection system (Applied Biosystems) using the dissociation protocol. The real-time PCR reactions components were 2 μl SYBR Green PCR Master Mix (Applied Biosystems), 3 μl each forward and reverse primers (5 μM), 2 μl plasmid or DNA, adjusted with Nuclease Free Water to 50 μl. The real-time PCR conditions were an initial 2 min at 50°C followed by 10 min at 95°C and 40 or 50 repetitions of 95°C for 15 s and 60°C for 1 min (for the 1.715 satellite and the SRY gene respectively). Each run included standard dilutions of plasmids containing the cloned fragments of the 1.715 satellite region or the SRY gene from which the primers were designed. The standard dilutions ranged from 2,280,000,000 to 22,800 copies of the 1.715 satellite and 1,580,000 to 15 copies of the SRY gene. Each run also included a standard pool of 1.9 ng male somatic cell DNA. All samples were run in triplicate on 96 well plates and two wells per sample were

used for the analysis of each run.

E. Validation of Somatic Cell DNA Removal and Sperm DNA Extraction

The effectiveness of the procedure to remove somatic cell DNA from sperm samples was validated using *in vitro* cultured oviductal cells. Confluent oviductal cells were dissociated using 1ml of trypsin/EDTA. Cells from 3 cows were combined and counted on a hemacytometer. Media was added to adjust the final concentration of the oviductal cells to 10,000/μl. The method for removal for somatic cell DNA removal and sperm DNA extraction described above was used on the following samples: 10,000 oviductal cells, 100,000 oviductal cells, 100,000 sperm cells. After DNA extraction, the 1.715 satellite copy number was determined for each sample using the real-time PCR assay described above. A sample of female somatic cell DNA (10pg/μl) was included in the real-time PCR run as a control.

F. Assay Validation

Repeatability was evaluated using male somatic cell DNA as a template. Inter- and intra-assay CVs were used to verify the repeatability of the real-time PCR assays. Two wells of 1.9 ng of male somatic cell DNA were amplified in each of two real-time PCR assays for each primer to determine inter- and intra-assay CVs.

The specificity of each primer set was determined using male and female somatic cell DNA. Copies of the 1.715 satellite and the SRY gene were

determined for 4 wells each of 20 ng, 2 ng, and 0.2 ng of female DNA and 19 ng, 1.9 ng, and 0.19 ng of male DNA in 2 separate real-time PCR runs. Copies per ng DNA were compared between male and female DNA for the 1.715 satellite and the SRY gene.

The accuracy of the assay in quantifying numbers of Y sperm was evaluated using samples of sorted sperm with known amounts of Y sperm. DNA was extracted from sperm samples sorted to contain 16.8%, 32.0%, 66.5%, and 79.0% Y sperm (donated by XY Inc.). Before DNA extraction, samples contained 1.5×10^6 sperm, 0.6×10^6 sperm, 0.75×10^6 sperm and 1.6×10^6 sperm for each concentration, respectively. Copies of SRY gene and 1.715 satellite were determined in duplicate samples using the above-described primer sets in 2 real-time PCR runs each. Accuracy of the assay was determined by comparing the copies of SRY gene observed for each sample with the copies of SRY gene expected for each sample. The copies of SRY gene expected for each sample were determined using the following equation:

$$\text{SRY expected} = \text{SRY expected per Y sperm} \times \frac{\text{Y sperm}}{\text{well}} \times \%\ \text{Recovery}$$

Where SRY expected is the expected copies of SRY gene and SRY expected per Y sperm is the copies of SRY gene expected per Y sperm cell. This equation was derived using several equations that follow. The expected copies of SRY gene per Y sperm was determined using the following equations:

$$\text{SRY expected per Y sperm} = \frac{\text{DNA}}{\text{Y sperm}} \times \frac{\text{Copies SRY gene}}{\text{pg DNA}}$$

$$\frac{\text{Copies SRY gene}}{\text{pg DNA}} = \frac{\text{SP copies SRY gene}}{\text{1.9 ng DNA}}$$

Where SP copies of SRY gene is the number of copies observed for the standard pool of DNA included in each run and the 1.9 ng DNA is the amount of DNA in each well of the standard pool of DNA. The amount of DNA in each Y sperm was 3.15 pg (Walker and Yates, 1952).

The number of Y sperm per well was determined based on the number of starting sperm and dilutions used for PCR.

$$\frac{\text{Y sperm}}{\text{well}} = \frac{\text{Y sperm}}{\mu\text{l}} \times \frac{2\ \mu\text{l}}{\text{well}}$$

$$\frac{\text{Y sperm}}{\mu\text{l}} = \frac{\text{Y sperm in sample}}{\text{Volume of sample}}$$

$$\text{Y sperm in sample} = \text{total sperm in sample} \times \%\ \text{Y sperm}$$

Where the total sperm in the sample was determined using a hemacytometer and the % Y sperm of each sample was given by XY Inc.

The percent recovery was determined for each sample using the results for the 1.715 satellite for each sample and the following equations:

$$\%\ \text{recovery} = \frac{\text{1.715 observed per well}}{\text{1.715 expected per well}} \times 100\%$$

$$\text{1.715 expected per well} = \frac{\text{DNA}}{\text{well}} \times \text{1.715 expected per pg DNA}$$

$$\frac{\text{DNA}}{\text{well}} = \frac{\text{DNA}}{\text{sperm}} \times \frac{\text{sperm}}{\text{well}}$$

$$\frac{\text{sperm}}{\text{well}} = \frac{\text{Total sperm in sample}}{\text{Total volume of sample}} \times \frac{2\ \mu\text{l}}{\text{well}}$$

$$\text{1.715 expected per pg DNA} = \frac{\text{SP copies 1.715}}{\text{1.9 ng DNA}}$$

Where 1.715 observed per well is the number of copies of 1.715 satellite observed per well of each sample and 1.715 expected per well is the number of copies of 1.715 satellite expected per well for each sample. 1.715 expected per pg DNA is the number of copies of the 1.715 satellite expected for each pg of DNA in the sample. SP copies 1.715 is the number of copies of 1.715 satellite observed for the standard pool of DNA included in each run and the 1.9 ng DNA is the amount of DNA in each well of the standard pool of DNA.

G. Statistical Analysis

Differences in copies of 1.715 satellite and SRY gene in male versus female genomic DNA were determined by Student's t-test. Differences in observed copies of SRY gene versus expected copies of SRY gene were determined by Student's t test.

IV. RESULTS

A. Somatic Cell DNA Removal and Sperm DNA Extraction

No copies of 1.715 satellite sequence were detected for the oviductal cell samples. An average of 99 million copies of 1.715 satellite sequence were detected in the sperm samples. An average of 0.69 million copies of 1.715 satellite sequence were detected in the female somatic cell DNA control.

B. Standard Curves

Standard curves were developed from plasmids containing the ligated

fragments of the 1.715 satellite and the SRY gene (Figure 2.2). Average R^2 for

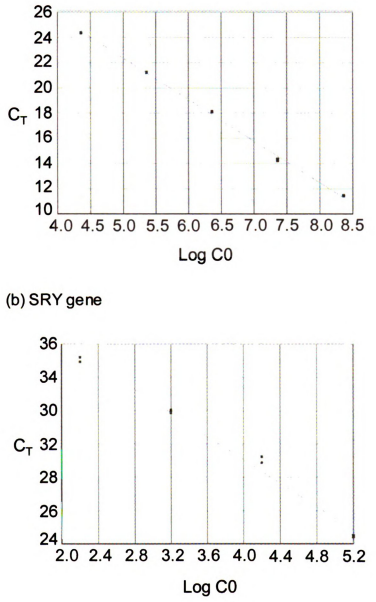


Figure 2.2: Representative standard curves for (a) the 1.715 satellite (R^2 = 0.9977) and (b) the SRY gene (R^2 = 0.9892); C_T = threshold cycles.

the standard curves were 0.9977 for the 1.715 satellite and 0.9892 for the SRY gene.

C. Coefficients of Variation

Inter- and intra-assay CVs determined with fixed amounts of male genomic DNA were 2.17% and 1.54% for the 1.715 satellite and 8.79% and 9.08% for the SRY gene, respectively (Table 2.2).

Table 2.2 : Inter- and Intra-Assay Coefficients of Variation

Sequence	Inter-Assay CV	Intra-Assay CV
1.715 Satellite	2.17%	1.59%
SRY Gene	8.79%	9.08%

D. Primer Specificity

Similar copy numbers per unit DNA were obtained for the 1.715 satellite sequence in both male and female somatic cell DNA samples (Figure 2.3). The SRY sequence was not detected in female somatic cell DNA samples (Figure 2.4).

E. Accuracy of the Assay in Quantification of SRY Copy Number

No differences in observed versus expected copies were detected for any sample of sorted sperm (Figure 2.5). It should be noted that the samples with higher percent Y do not necessarily have a greater copy number of the SRY

gene. This is because the samples had different starting number of sperm as well as different percent recovery for the DNA extraction.

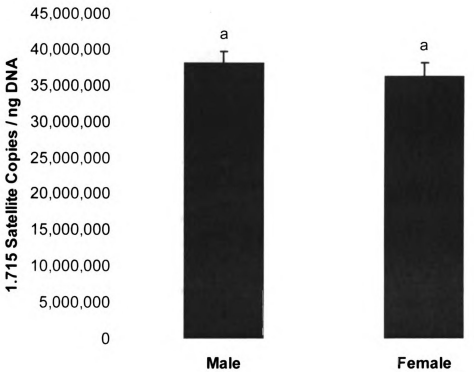


Figure 2.3: Quantitative real time PCR analysis of copies of 1.715 satellite sequence in samples of male and female somatic cell DNA. DNA was extracted from male (liver) and female (corpus luteum) tissue samples and copies of the 1.715 satellite sequence were determined in duplicate samples. Data are expressed as copies of 1.715 satellite DNA per ng genomic DNA and are depicted as mean ±SEM. Means with common superscripts are not different.

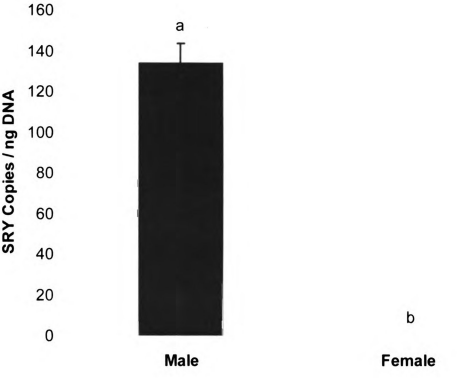
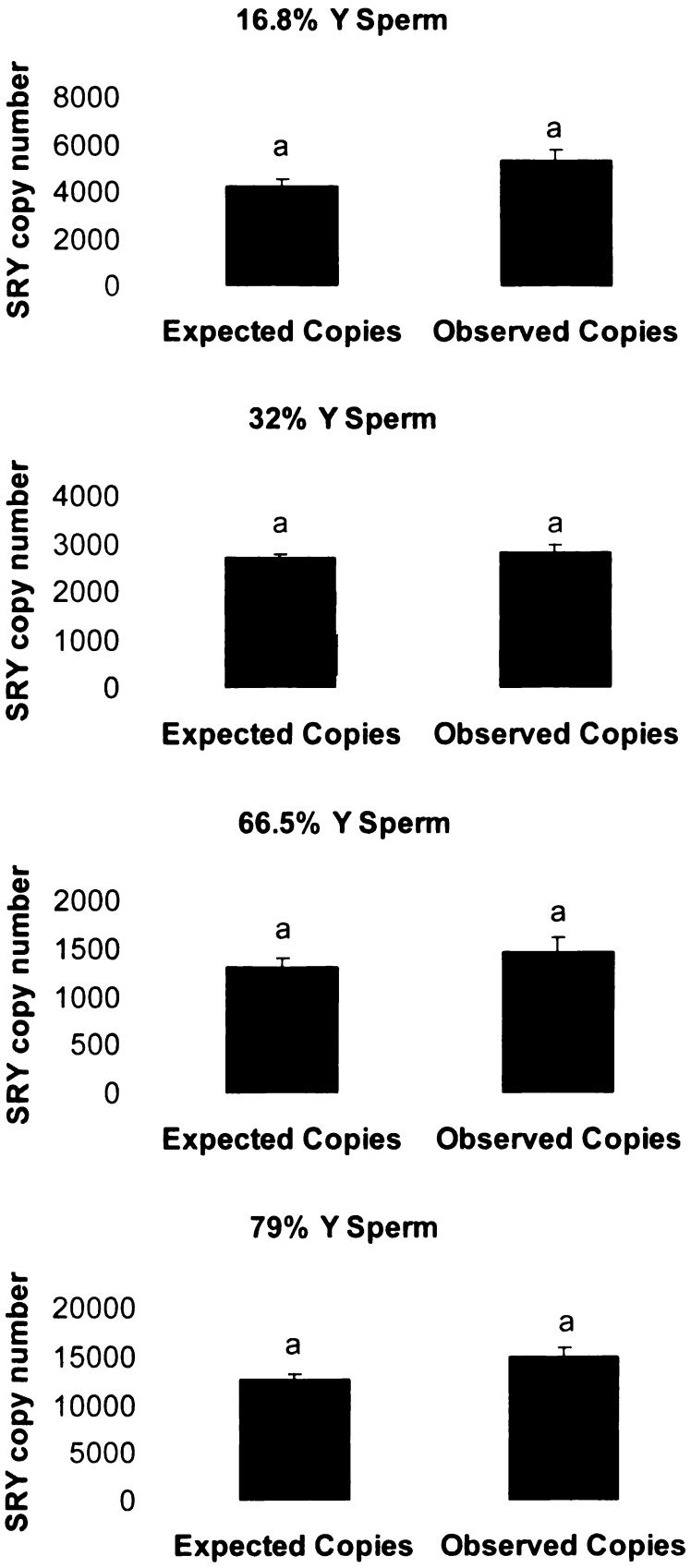


Figure 2.4: Quantitative real time PCR analysis of copies of SRY gene in male and female somatic cell DNA. DNA was extracted from male (liver) and female (corpus luteum) tissue samples and copies of the SRY gene were determined in duplicate samples. Data are expressed as copies of SRY gene per ng genomic DNA and depicted as mean $\pm$ SEM. Means without a common superscript are different at $P < 0.001$.

Figure 2.5: Comparison of expected copies of SRY gene versus observed copies of SRY gene in samples of semen separated by flow cytometry. Samples of separated sperm with known amounts of X and Y sperm were used to determine accuracy of the quantitative real-time PCR assay in quantification of numbers of Y-sperm. DNA was extracted from sorted sperm samples containing (a) 16.8%, (b) 32%, (c) 66.5%, and (d) 79% Y-sperm and copies of SRY and 1.715 satellite DNA determined in duplicate samples. Percent recovery of genomic DNA from sorted sperm samples was calculated using results of 1.715 satellite assay and used to adjust expected number of SRY copies for each sample of sorted sperm analyzed. Differences in observed versus expected copies of SRY gene were determined by Student's t test. Data are expressed as copies of SRY and are depicted as mean ± SEM. Means with common superscripts are not different.

Figure 2.5


16.8% Y Sperm
SRY copy number
8000
6000
4000
2000
0
a
a
Expected Copies
Observed Copies
32% Y Sperm
SRY copy number
4000
3000
2000
1000
0
a
a
Expected Copies
Observed Copies
66.5% Y Sperm
SRY copy number
2000
1500
1000
500
0
a
a
Expected Copies
Observed Copies
79% Y Sperm
SRY copy number
20000
15000
10000
5000
0
a
a
Expected Copies
Observed Copies

V. DISCUSSION

X and Y sperm can be distinguished based on differences in DNA content or the presence of specific genes on the X or Y chromosomes. To date, only a handful of techniques have been reported that can utilize these differences for quantification of X and Y sperm in a research or commercial setting. The chromosome content of individual sperm can be visualized and karyotyped in a system that uses zona free hamster oocytes to de-condense sperm chromatin (Tateno and Mikamo, 1987). However, this method is costly and time consuming. FISH can be used to identify and quantify X and Y sperm (Hassanane, et al., 1999, Piumi, et al., 2001, Rens, et al., 2001). Technical difficulty and the requirement of microscopy and visualization of individual sperm make this technique time consuming and low throughput. Flow cytometry is a well-established method for sorting sperm (Pinkel, et al., 1982, Garner, et al., 1983, Pinkel, et al., 1985, Johnson, et al., 1987a, Welch and Johnson, 1999). However, specialized and expensive equipment are needed (Pinkel, et al., 1982, Rens, et al., 1998, Johnson, et al., 1999, Johnson, 2000, Garner, 2006). Methods for quantification of the proportion of Y sperm by PCR coupled with gel or capillary electrophoresis have also been reported (Chandler, et al., 1998, Checa, et al., 2002). However these methods are only semi-quantitative because they rely on detection after the PCR reaction is complete and, therefore, cannot account for the slowing of the reaction as the substrates are used. Parati et. al. (2006) recently reported a method to determine sex ratio in bovine semen using real-time PCR. In this method, two sets of primers and internal TaqMan probes were

designed on specific X- and Y-chromosome genes. This method was demonstrated to be both rapid and reliable (Parati, et al., 2006). This method had not been published at the time the real-time PCR assay described above was developed.

The real-time PCR assay developed was shown to be reliable and repeatable in determining the number of Y sperm in samples from cattle. This assay was developed in order to test if the proportion of X and Y bearing sperm attached to in vitro cultured oviductal cells changes over time (Chapter Three). While most of the techniques described above were available when the methods of described in this chapter were developed, they did not meet the needs of the study due to the limitations discussed. It was determined that in order to test the objective of Chapter Three, a real-time PCR based assay needed to be developed.

Sex preselection of livestock offspring in cattle represents a big potential for genetic improvement (Parati, et al., 2006). The separation of X and Y sperm provides a tool for production of a specific sex of offspring. The only proven method currently available to sort X and Y sperm is cell sorting by flow cytometry (Johnson, et al., 1987b). However, the method used to verify the sorting of the samples is currently flow cytometry reanalysis (Welch and Johnson, 1999). The real-time PCR assay developed could be a useful tool for validation of sexed semen samples using a method that does not rely on the same instrumentation used to sort the samples. Sorting semen using flow cytometry requires the use of a DNA binding dye and laser excitation, which may be harmful to sperm and

result in decreased fertility or abnormal offspring (Seidel, 2003). Flow sorting of semen is also limited by the number of sperm that can be sexed in a given time (Seidel, 2003). Because of these limitations, other methods to sex semen are currently of interest. The real-time PCR assay developed here could provide a means to validate other techniques developed to separate X and Y sperm. Further, the assay could be used to determine the outcomes of experiments designed to find differences in X and Y sperm. Any differences in X and Y sperm, even small differences, may someday prove useful for separating sperm.

VI. CONCLUSION

The results indicate the above-described quantitative real-time PCR assay is reliable and repeatable in determining numbers of Y sperm in samples. This assay will be utilized in future studies (Chapter Three).

CHAPTER THREE

Differences in X- and Y-Chromosome Bearing Sperm Attachment to *In Vitro* Cultured Oviductal Epithelial Cells Over Time

I. ABSTRACT

Recent data indicate that increasing the time from insemination to ovulation inversely affects the subsequent sex ratio of calves (Pursley, et al., 1998, Macfarlane, 2003). Deviation of X and Y sperm over time in favor of greater numbers of X sperm is one possible explanation for this phenomenon. *In vivo* sperm form a reservoir in the oviductal isthmus by attaching to epithelial cells. Attachment to oviductal cells in the reservoir preserves sperm fertility during the pre-ovulatory period. Differences in the abilities of X and Y sperm to remain attached to oviductal epithelial cells over time may influence the proportion of X and Y sperm available for fertilization. The objective of this study was to test if the proportion of X and Y sperm attached to *in vitro* cultured oviductal epithelial cells changes over time. Sperm were added to in vitro cultured oviductal epithelial cells and allowed to attach. The unattached sperm were removed 2 h after addition of sperm to the oviductal epithelial cells. The sperm that attached to the oviductal epithelial cells were incubated for a total of 12, 24, or 36 h. The real-time PCR assay described in Chapter Two was used to determine the percent Y sperm in the samples of attached sperm. Proportion of Y sperm attached to oviductal cells consisted of 41.01 ± 1.73%, 48.37 ± 1.89%,

33.59 ± 1.49%, and 41.30 ± 1.59% (mean ± SEM) Y sperm at 2, 12, 24, and 36 h, respectively. Percent Y sperm decreased significantly from 12 to 24 h ($P < 0.05$). In summary, these *in vitro* data indicate that X and Y sperm differ in the amount of time they are able to remain attached to oviductal epithelial cells. Thus, changes in sex ratio due to extending time from AI to ovulation may be the result of differences in the ability of X and Y sperm to remain attached to oviductal cells during the pre-ovulatory period.

II. INTRODUCTION

The ability to predetermine sex of livestock could increase the efficiency of agricultural production, reduce animal production costs and provide a means to control and eliminate undesirable sex-linked traits (Gledhill, 1988, Windsor, et al., 1993, Hossain, et al., 1998, Rorie, 1999, Seidel and Johnson, 1999). Physiological mechanisms that may influence sex ratio include facilitating or inhibiting transport of either X or Y sperm, preferential selection of sperm at fertilization, or sex-specific embryo death (Pursley, et al., 1998, Rosenfeld and Roberts, 2004). Differences between X and Y sperm in timing of capacitation or survival could also affect the sex of the offspring produced (Wehner, et al., 1997, Gutierrez-Adan, et al., 1999, Lechniak, et al., 2003, Martinez, et al., 2004, Roelofs, et al., 2006).

Time of insemination relative to ovulation influenced sex of offspring (Pursley, et al., 1998, Macfarlane, 2003). Early insemination produced more female calves (Pursley, et al., 1998, Macfarlane, 2003). When cows were

inseminated 24 to 32 h before ovulation 61% females were produced (Pursley, et al., 1998). In a follow-up study, cows inseminated 32 to 40 h before ovulation produced 65% females while cows inseminated 8 to 16 h before insemination produced 56% females (Macfarlane, 2003).

Early insemination increases the time from insemination to ovulation and, therefore, the time sperm spend in the female reproductive tract before fertilization. *In vivo*, sperm form a reservoir in the oviductal isthmus by binding to oviductal epithelial cells (Suarez, 1998, Suarez, 2002, Hunter, 2003). Binding to oviduct cells preserves sperm fertility over time (Pollard, et al., 1991). Sperm are sequestered in the caudal isthmus for most of the pre-ovulatory period (Hunter and Wilmut, 1984). Around the time of ovulation sperm capacitation is triggered and sperm are released from the reservoir (Lefebvre and Suarez, 1996, Suarez, 1998, Topfer-Petersen, 1999b, Revah, et al., 2000, Suarez, 2002, Gualtieri, et al., 2005). The membrane changes of capacitation cause sperm to lose binding affinity for oviductal cells and gain the ability to fertilize an oocyte (Lefebvre and Suarez, 1996, Suarez, 1998, Topfer-Petersen, 1999b, Revah, et al., 2000, Suarez, 2002, Gualtieri, et al., 2005).

One explanation for the effect of insemination time on sex ratio is that X and Y sperm may differ in capacitation times (Wehner, et al., 1997, Martinez, et al., 2004, Roelofs, et al., 2006). If Y sperm became capacitated before X sperm they would be released from the reservoir earlier and would be the first sperm available for fertilization (Martinez, et al., 2004). However, capacitated sperm do not remain viable for long (Soupart and Orgebin-Crist, 1966, Bedford, 1970).

Thus, if insemination occurred earlier Y sperm would reach the site of fertilization long before ovulation (Martinez, et al., 2004). X sperm would become capacitated later and therefore have a longer lifespan. Therefore, the X sperm would be released from the reservoir closer to the time of ovulation and be favored for fertilization (Martinez, et al., 2004).

There is also *in vitro* evidence for differences between X and Y sperm. Sperm pre-incubated in sperm-TALP at 39°C for 24h prior to IVF produced more female hatched-blastocysts compared to 0 or 6 h of incubation. These results indicated that X sperm remained viable longer than Y sperm (Lechniak, et al., 2003). In other studies, the maturational state of the oocyte had an effect on the sex ratio of the resulting embryos (Dominko and First, 1997, Gutierrez-Adan, et al., 1999). *In vitro* matured oocytes were fertilized immediately after the first PB was extruded (immature) or 8 h later (mature). Immature oocytes produced more female embryos while mature oocytes produced more male embryos (Dominko and First, 1997, Gutierrez-Adan, et al., 1999). This could be explained by intrinsic differences in the physiological activity of X and Y sperm (Gutierrez-Adan, et al., 1999). Predominantly male offspring could be produced if Y sperm reacted with capacitation or changes in motility or viability earlier following insemination (Gutierrez-Adan, et al., 1999). The early reaction of Y sperm implies that when immature oocytes are used, Y sperm die earlier (before the oocyte is receptive to fertilization) and a higher proportion of X sperm survive and are available for fertilization (Gutierrez-Adan, et al., 1999). Similarly, a short sperm-oocyte co-incubation time (6 h) during IVF produced more males, while extending the co-

incubation time produced equal numbers of males and females (Kochhar, et al., 2003). These data indicated that during the first 6 h of co-incubation Y sperm had an advantage for fertilization that was lost during extended co-incubation.

Our hypothesis is that X and Y sperm deviate over time, such that X sperm have an advantage in remaining attached to the oviductal epithelium for longer periods. This advantage may be responsible for the preponderance of females seen when the time from insemination to ovulation is increased. Our specific aim was to determine if the proportion of X and Y sperm that remained bound to *in vitro* cultured oviductal epithelial cells changed over time.

III. MATERIALS AND METHODS

A. Oviductal Cell Collection and Culture

Oviductal epithelial cells were collected and cultured based on previously described methods (Bosch, et al., 2001). Oviducts were collected at a slaughterhouse (Packerland; Plainwell, MI) and transported on ice to the laboratory in less than 2 hours. Only those oviducts from reproductive tracts with ovaries that contained no functional corpus luteum and at least one large follicle (>10 mm) were selected. These criteria were used to obtain oviductal cells from cows that were in the pre-ovulatory phase of the estrous cycle in order to reduce possible variation among cells. Oviducts were dissected free from surrounding tissue and clamped at the UTJ and just below the infundibulum. Clamped oviducts were immersed in 70% ethanol, followed by two rinses in D-PBS without Ca^{2+} and Mg^{2+} (Invitrogen Corporation; Carlsbad CA). Oviducts were injected

with collagenase (0.1% w/v in D-PBS; Sigma Chemical Company; St. Louis, MO) and incubated at room temperature for 30 min. After incubation, oviducts were unclamped and flushed with 10 ml Ham's F-12 nutrient medium (Invitrogen Corporation; Carlsbad CA) containing 10% fetal bovine serum (FBS; HyClone; Logan, TX), 10 μg/ml ciprofloxacin (Mediatech, Inc; Herndon, VA), and 0.25 μg/ml fungizone (Invitrogen Corporation; Carlsbad, CA). The media was collected in 50 ml conical tubes after flushing. Both oviducts of a tract were flushed into the same tube. Tubes were centrifuged for 10 min at 200 x g and the supernatant was discarded. The cell pellet was suspended in 12 ml of F-12 containing 10% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 10 ng/ml epidermal growth factor (EGF; Sigma Chemical Company; St. Louis, MO), 10 μg/ml ciprofloxacin, and 0.25 μg/ml fungizone. Cell suspensions were plated in 25 cm^2 tissue culture flasks (Corning Incorporated; Corning, NY) and grown in an atmosphere of 5% CO_2 in air at 38.5°C until confluent. Media was replaced every other day. After 2 weeks of culture, the same media without antibiotic or antifungal was used. Upon reaching confluence, morphologically normal cells were dissociated by incubating with 1 ml trypsin/EDTA for 5 min. Dissociated cells from at least 3 cows were combined and plated onto 12 wells of each 24 well plate (Corning Incorporated; Corning, NY) and grown to confluence.

B. Sperm Preparation

Bull semen was donated by Alta Genetics (Alberta, Canada). For each replicate, two straws from each of 8 bulls (labeled 1-8) were removed from liquid

nitrogen and thawed in a 37°C water bath for 60 s. Semen was pooled for each bull and placed on a 30/60/90 Percoll gradient (Sigma Chemical Company; St. Louis, MO) to separate live sperm. The different concentrations for the Percoll gradients were made by combining Percoll with sperm-TALP. Sperm-TALP consisted of 5.8 mg/ml NaCl, 2.09 mg/ml $NaHCO_3$, 2.38 mg/ml HEPES, 40 μg/ml NaH_2PO_4, 10 μg/ml Phenol Red, 4 μg/ml sodium lactate, 310 μg/ml $MgCl_2.6H_2O$, 384 μg/ml $CaCl_2.2H_2O$, 6mg/ml BSA, 110 μg/ml sodium pyruvate and 50 μg/ml gentamicin sulfate. For replicates 3 and 4 a 30 μl sample of sperm was removed before the Percoll gradient to determine the percent Y of the starting sperm. After separation the live sperm were washed in sperm-TALP and centrifuged at 114 x g for 10 min. The supernatant was removed, leaving a sperm pellet in approximately 0.5-0.6 ml of sperm-TALP. Sperm concentration was determined with a hemacytometer. The volume of the sperm suspension was adjusted to 3 ml using IVF media. IVF media consisted of 6.66 mg/ml NaCl, 2.1 mg/ml $NaHCO_3$, 235 μg/ml KCl, 47 μg/ml NaH_2PO_4, 10 μg/ml Phenol Red, 65 μg/ml penicillin, 1.86 mg/ml sodium lactate, 100 μg/ml $MgCl_2.6H_2O$, 397 μg/ml $CaCl_2.2H_2O$, 6 mg/ml BSA, 22 μg/ml sodium pyruvate. Media in 12 wells of each of 4 24-well plates (labeled 2h, 12h, 24h, and 36h) containing confluent oviductal epithelial cells was replaced with 0.5 ml of sperm suspension. Only 12 of the 24 wells in each plate were used because the amount of time required for the procedures would only allow a maximum of 12 wells to be processed at a time. For all replicates each bull was represented on each plate and repeated on 2 plates. Each bull had a total of 6 wells for each incubation time over all replicates.

Table 3.1: Bovine Sperm Arrangement Across Replicates													
		Well											
Replicate	**Plate**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	**2h**	3	8	2	7	5	1	6	5	4	1	4	6
	12h	4	7	5	3	2	8	1	8	2	6	7	3
	24h	6	2	1	5	8	4	8	3	7	4	6	5
	36h	5	4	7	1	3	7	3	2	6	8	1	2
2	**2h**	8	6	3	4	7	2	2	7	1	5	3	8
	12h	1	3	8	6	4	6	7	4	5	2	5	1
	24h	2	5	6	8	1	3	4	1	3	7	2	7
	36h	7	1	4	2	6	5	5	6	8	3	8	4
3	**2h**	4	3	1	5	6	4	8	6	2	7	8	2
	12h	8	2	7	1	7	5	5	3	4	5	3	1
	24h	7	4	3	6	1	2	2	5	1	8	7	8
	36h	6	1	2	8	3	6	7	4	3	5	4	5
4	**2h**	5	8	6	2	5	7	4	1	7	3	1	3
	12h	3	6	5	4	2	8	1	7	8	2	6	4
	24h	1	7	8	3	4	3	6	2	5	4	5	6
	36h	2	5	4	7	8	1	3	8	6	1	2	7

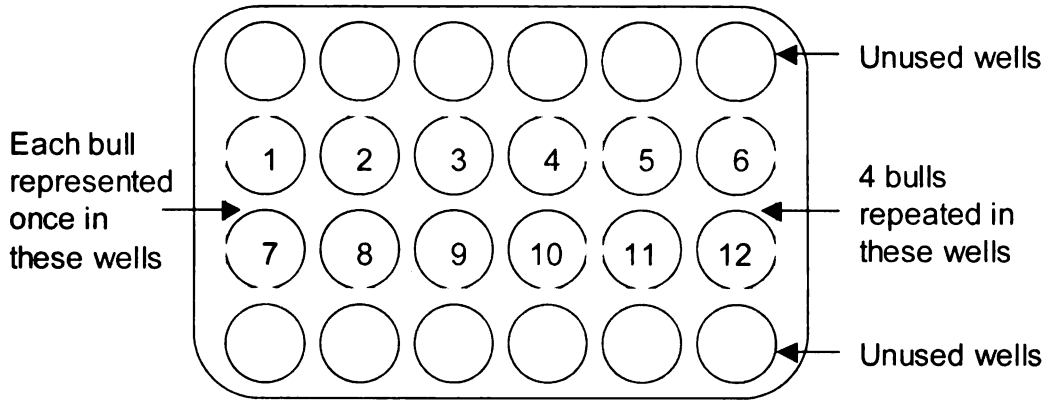


Figure 3.1: Arrangement of wells used for sperm-oviduct co-incubation. Oviductal cells were grown to confluence in 12 wells of each 24 well plates (numbered 1-12). Four plates were used for each of 4 replicates, one plate for each incubation time (2, 12, 24, and 36 h). Sperm form 8 different bulls was placed on the oviductal cells in each well. Sperm from each bull was placed on wells 1-4 and 7-10 so that each bull was represented once on each plate. Sperm from 4 of the bulls was also placed in wells 5, 6, 11, and 12 so that half the bulls were repeated on each plate. The bulls that were repeated varied among the plates and replicates so that each bull was represented in a total of 6 wells for the entire experiment (Table 3.1).

Bull placement was determined beforehand and varied among plates as well as among replicates to avoid any positional biases (Table 3.1 and Figure 3.1). Sperm and oviductal cell co-cultures were incubated in an atmosphere of 5%

CO_2 in air at 38.5°C.

C. Preliminary Experiments to Determine Methods for Sperm Co-Incubation and Removal

Media for Co-Incubation of Sperm with Oviductal Cells

Different medias were compared to determine the effect on the oviductal cells. Oviductal cells were cultured to confluence in 24 well plates as described above. Media was removed from 3 wells of confluent oviductal cells and replaced with 0.5 ml of sperm-TALP, IVF media or F-12 based media used for culturing oviductal cells. Cells were incubated at 38.5°C. Cells were visualized by microscopy at 0, 2, 12, 24, and 36 h to compare cell survival. Cells were considered healthy when they remained confluent and looked morphologically normal for epithelial cells. Cells were considered unhealthy or dead when they became dissociated from the plate, floated in the media, and looked round.

Removal of Unattached Sperm

A preliminary experiment was conducted to determine the best time to remove unattached sperm in order to maximize the number of attached sperm. Five straws of semen were thawed and live sperm were separated on a Percoll gradient as described above. Live sperm from all straws were combined and the concentration was determined using a hemacytometer. IVF media was added to dilute the sperm suspension to a final concentration of 4×10^6 sperm / ml. The media was removed from confluent oviductal cells on 24 well plates and 0.5 ml of

sperm suspension was added to each of 20 wells (2 x 10^6 sperm / well). Sperm and oviductal cells were incubated for 1, 2, 3, 4, or 5 h at 38.5°C. At each incubation time unattached sperm were recovered from 4 wells by pipetting the media up and down 5 times. The number of unattached sperm recovered was determined for each well using a hemacytometer. Differences between incubation times were determined using Fisher's LSD. Differences were considered significant at $P < 0.05$.

Removal of Attached Sperm

The optimal time for incubation of sperm with heparin was determined. Sperm were thawed and live sperm were separated on a Percoll gradient as described above. The number of live sperm was determined and IVF media was added to a final concentration of 4 x 10^6 sperm / ml. The actual concentration of the sperm suspension after adjustment was determined on a hemacytometer. The media was removed from 12 wells of confluent oviductal cells and replaced with 0.5 ml of sperm suspension. Sperm were co-incubated with oviductal cells for 2 h at 38.5°C. Unattached sperm were removed at 2 h by pipetting media up and down 5 times. Number of unattached sperm recovered was determined for each well using a hemacytometer. After removal of unattached sperm, 0.5 ml of sperm-TALP containing 200μg/ml was added to each well and incubated for 15 or 30 min (6 wells each). Sperm were removed from oviductal cells after heparin incubation the sperm-TALP was collected, wells were washed with fresh sperm-TALP, which was also collected and combined with the first collection. The

number of sperm recovered after heparin was determined using a hemacytometer. The number of sperm that remained in each well was determined indirectly by calculating the number of sperm recovered from each well and subtracting the number recovered from the number of sperm placed in each well. The percent sperm left was determined by dividing the number of sperm remaining by the number of sperm placed on each well. The percent sperm left after 15 min heparin incubation was compared to the percent sperm left after 30 min heparin incubation using a Student's t-test. The difference was considered significant at $P < 0.05$.

Preliminary experiments were conducted in order to determine the approximate amount of sperm left on the oviductal cells after heparin removal of unattached. Sperm were incubated with oviductal cells (6 wells), unattached sperm were removed after 2h, and attached sperm were removed by incubation with heparin for 30 min as described above. Sperm remaining on the oviductal cells were visualized with a microscope (200x). The number of sperm attached to oviductal cells was counted in 6 visual fields of each well. One visual field was determined to have an area of 0.64326 mm^2. The average number of sperm per mm^2 was determined and the total number of sperm per well was estimated by multiplying the average number of sperm per m m^2 for each well by the area of each well (78.54mm^2).

D. Sperm Co-Incubation and Removal

Figure 3.2 depicts the experimental design for sperm coincubation and

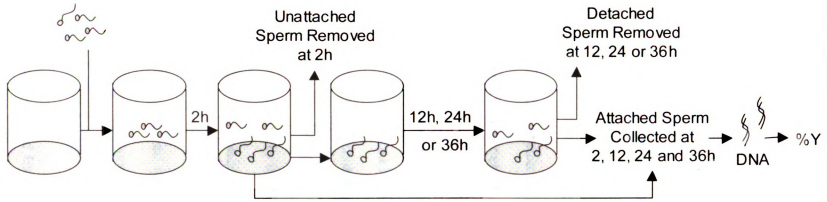


Figure 3.2: Experimental Design. Sperm were added to confluent oviductal epithelial cells on 4 plates (12 wells per plate) and allowed to attach to the oviductal cells for 2h. After 2h of co-incubation, unattached sperm were removed from all plates and attached sperm were collected from one plate. The remaining plates were incubated for 12h, 24h, and 36h total. After incubation, detached sperm were removed and attached sperm were collected. DNA was isolated and extracted from the collected sperm and Y sperm was determined.

removal. Sperm were allowed to attach to the oviductal cells for 2h. After 2h, unattached sperm were removed from all plates by pipetting the media up and down 5 times. The media was replaced with fresh IVF media (12h, 24h, and 36h plates) or sperm-TALP containing 200 μg/ml of heparin (2h control plate; Sigma Chemical Company; St. Louis, MO). Plates were checked under the microscope to confirm oviductal cells had not been removed by the procedure and returned to the incubator. After 30 min the 2h plate was removed and the sperm collected. Many sperm were detached and free in the media, the remaining sperm were removed by pipetting the media up and down 5 times. The media of each well was squirted gently onto the cells 5 times to dislodge any remaining sperm. The sperm suspension was placed into a 1.5 ml microcentrifuge tube and the washing step was then repeated using D-PBS. The sperm from the second wash was placed in the same tube as the first. The remaining plates were incubated for 12, 24, or 36 h total. After incubation, detached sperm were removed from the plate by pipetting the media up and down 5 times. Sperm-TALP containing 200 μg/ml of heparin was added to the plate and incubated for 30 min to remove the attached sperm from the oviductal cells. Prior to placing the plate back in the incubator plates were checked on the microscope to verify that the oviductal cells were still intact. Wells that had disrupted oviductal cells were excluded from the collection. Attached sperm were collected from the 12, 24, and 36 h plates as described above for the 2 h plate.

E. Sperm DNA Isolation and Extraction

Sperm were isolated from the few oviductal cells present in the samples

using an Epithelial Lysis Mixture as described in Chapter Two. Validation of the procedure is presented in detail in Chapter Two. Sperm were lysed and DNA was extracted using the method described in Chapter Two.

F. Real-Time PCR

Copies of the 1.715 satellite and the SRY gene were determined for each sample using the real-time PCR assay described in Chapter Two. Serial dilutions were made for the sperm DNA samples from each well. The dilution that amplified closest to the center of the standard curve for each primer set was used for the real-time PCR run. Samples were run using the same PCR conditions described in Chapter Two. Each run included standard dilutions of plasmids containing the cloned fragments of the 1.715 satellite region or the SRY gene from which the primers were designed. The standard dilutions ranged from 2,280,000,000 to 22,800 copies of the 1.715 satellite and 1,580,000 to 15 copies of the SRY gene. Each run also included a standard pool of 1.9 ng male somatic cell DNA. All samples were run in triplicate on 96 well plates and two wells per sample were used for the analysis of each run. The standard pool copy number was used to determine the CVs for each run.

G. Percent Y Sperm Calculation

Percent Y sperm for each sample was determined using the following equation:

$$\% \text{ Y sperm} = \frac{\frac{\text{SRY copies per sample}}{\text{SRY copies per SP}}}{\frac{\text{1.715 copies per sample}}{\text{1.715 copies per SP}}} \times \frac{3.15}{7.52}$$

Where SRY copies per sample is the number of copies of the SRY gene determined by the PCR assay for each sample and SRY copies per SP is the number of copies of the SRY gene determined by the PCR assay for the SP. The number of copies of the 1.715 satellite determined by the PCR assay for each sample is called 1.715 satellite copies per sample and the number of copies of the 1.715 satellite determined by the PCR assay for the SP is called 1.715 satellite copies per SP. Because the SRY gene is a single copy gene and the 1.715 sequence has multiple copies per cell the copy number of the SP was used to normalize the data. The correction factor of 3.15 / 7.52 was calculated based on the amount of DNA in each sperm (3.15 pg) and the amount of DNA in each somatic cell (7.52 pg) determined by Walker and Yates (1952).

H. Statistical Analyses

Percent Y sperm data were analyzed using the PROC MIXED function of SAS. Least Squares Means analyses were used to determine the effect of co-incubation time on percent Y sperm. A Tukey-Kramer adjustment was applied to the comparisons of percent Y sperm between each time point. Statistical significance was acknowledged when $P < 0.05$.

IV. RESULTS

A. Preliminary Experiments to Determine Methods for Sperm Co-Incubation and Removal

Media for Co-Incubation of Sperm with Oviductal Cells

At both 0 and 2 h all cells look confluent with no signs of degradation or cell death. At 12 h cells in culture media and IVF media are still confluent and look healthy. However, cells in sperm-TALP are starting to dissociate from the plate. Cells in culture media and IVF media continued to look confluent and healthy at 24 and 36 h. Most cells in sperm-TALP were dissociated and dead by 24 h and very few healthy-looking cells were seen at 36 h. IVF media was chosen for sperm co-incubation with oviductal cells.

Removal of Unattached Sperm

The number of unattached sperm recovered at each time is depicted in

Table 3.2: Unattached Sperm Recovered at Different Times	
Time of Co-Incubation (h)	Unattached Sperm Recovered (mean ± SEM)
1	$630{,}000 \pm 39{,}000^{ab}$
2	$510{,}000 \pm 36{,}000^{a}$
3	$750{,}000 \pm 81{,}000^{bc}$
4	$810{,}000 \pm 66{,}000^{bc}$
5	$920{,}000 \pm 37{,}000^{c}$
Means without common superscripts are different at $P < 0.05$	

Table 3.2. The lowest number of unattached sperm was recovered at 2h and was significantly different from the number of sperm recovered at 3, 4, and 5 h ($P < 0.05$). There was not a significant difference between the numbers of unattached

sperm recovered at 1 and 2 h ($P > 0.1$). The lower number of unattached sperm indicates there were more sperm attached at this time. For the main experiments, unattached sperm were removed after 2 h of co-incubation with oviductal cells.

Removal of Attached Sperm

The percent sperm left after 15 or 30 min heparin incubation was 44.03 ± 17.98% and 27.48 ± 11.22%, respectively (mean ± SEM). Percent sperm left was significantly less after 30 min of heparin incubation ($P < 0.05$). 30 min incubation with heparin was used to recover attached sperm at 2, 12, 24, and 36 h.

It was determined that 925,000 sperm were placed on each well using a hemacytometer. The number of sperm calculated to be left on each well was 1055 ± 175 (mean ± SEM). The sperm left on the cells after this method of removal was 0.11 ± 0.02% of the starting number of sperm. Less than 1% of sperm were left on the wells, indicating the method used was sufficient in removing sperm from oviduct cells.

B. Percent Y Sperm Attached to Oviductal Cells Over Time

Only those PCR reactions with CVs less than 20% were included in the statistical analysis. Samples that fell below the standard curve or that had CVs over 20% were excluded from the statistical analysis. Bull four was excluded from the analysis because oviductal cells became contaminated and died after co-incubation with semen from this bull. Replicate, bull, the number of starting sperm or the percent Y of the starting sperm did not affect percent Y sperm over

time (P > 0.1). Incubation time was the only factor that did have a significant effect (P < 0.05).

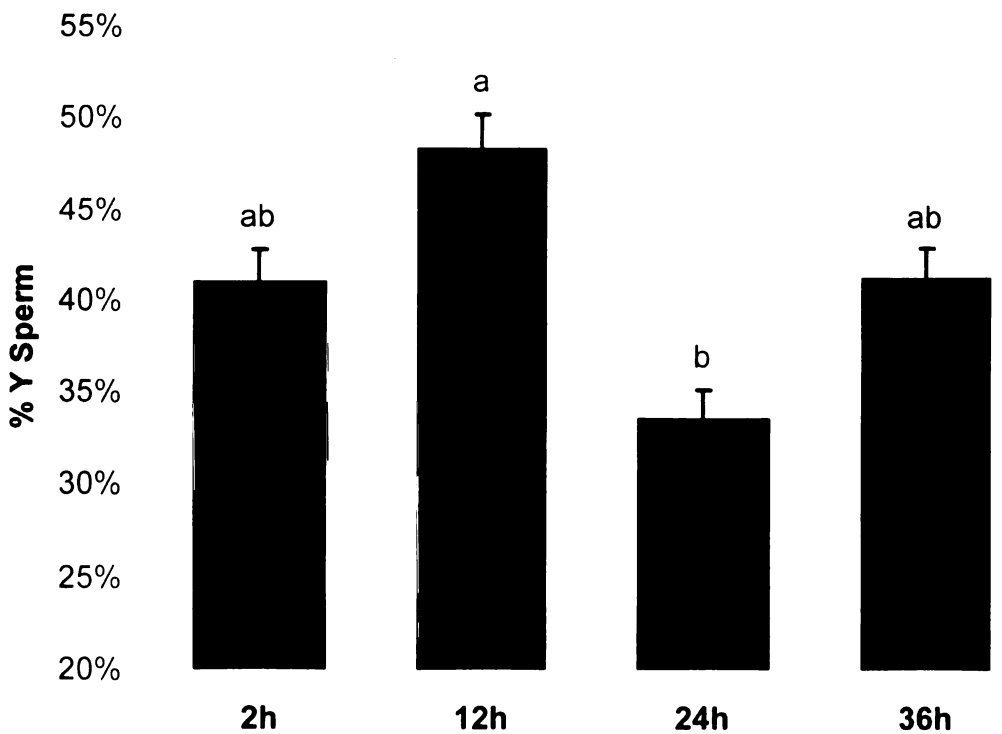


Figure 3.3: Changes in percent Y sperm attached to oviductal cells over time. Sperm were allowed to attach to *in vitro* cultured oviductal epithelial cells for 2h and were co-incubated for 12h, 24h, or 36h. Attached sperm were removed from the oviductal cells after co-incubation and percent Y sperm was determined. Differences in percent Y sperm between time points were determined using Least Squares Means analysis with a Tukey-Kramer adjustment. Data are expressed as percent Y sperm and depicted as mean ± SEM. Means without a common superscript are different at P < 0.05

Sperm attached to the oviductal cells after the initial two hours of co-incubation consisted of 41.01 ± 1.73% Y sperm (mean ± SEM). The sperm that remained attached for 12, 24, and 36 h consisted of 48.37 ± 1.89%, 33.59 ± 1.49%, and 41.30 ± 1.59% Y sperm, respectively (mean ± SEM). The percent Y sperm that were able to remain attached to the oviductal cells for 12, 24, and 36 h did not differ from the percent Y sperm that were able to attach within the first 2 h of co-incubation ($P > 0.1$). Percent Y sperm decreased from 12 to 24 h ($P < 0.05$) but was not different at 36 h than at either 12 or 24 h ($P > 0.1$). Average percent Y sperm at each co-incubation time are depicted in Figure 3.3.

V. DISCUSSION

There is some evidence that functional differences exist between X and Y sperm (Sarkar, et al., 1984, Dominko and First, 1997, Gutierrez-Adan, et al., 1999, Kochhar, et al., 2003, Lechniak, et al., 2003). This experiment indicated that there are differences between X and Y sperm that allow a greater proportion of X sperm to remain attached to oviductal cells over time. While many experiments have been conducted to determine differences between X and Y sperm, to our knowledge, differences in the ability of X and Y sperm to remain bound to oviductal cells over time had not been previously tested.

This study demonstrated differences between X and Y sperm in their ability to remain attached to *in vitro* cultured oviductal epithelial cells over time. The percent Y sperm decreased significantly from 12 to 24 h, indicating more X sperm remained attached to the oviductal cells during this time (Figure 3.3).

It was not surprising that the percent Y sperm decreased during the course of the experiment. Previous researchers demonstrated that Y sperm gain the ability to fertilize oocytes before X sperm (Dominko and First, 1997, Gutierrez-Adan, et al., 1999, Kochhar, et al., 2003). This early response of Y sperm may be due to early capacitation, which would also cause Y sperm to be released from oviductal cells earlier than X sperm (Gutierrez-Adan, et al., 1999). *In vitro* incubation of bull sperm in sperm-TALP at 39°C for 24 h before IVF produced more female hatched-blastocysts compared to incubation for 0 or 6 h (Lechniak, et al., 2003). This indicates that X sperm have longer functional survival or delayed capacitation compared to Y sperm (Lechniak, et al., 2003). IVF produced male embryos reached a more advanced stage of development than female embryos. One possible explanation for this is that Y sperm were able to fertilize the oocytes earlier than X sperm (Avery, et al., 1991, Avery, et al., 1992, Carvalho, et al., 1996, Beyhan, et al., 1999, Kochhar, et al., 2003). It has also been shown that short co-incubation times during IVF produce more male embryos and that extending co-incubation of sperm with oocytes produces equal numbers of male and female embryos (Kochhar, et al., 2003). One possible mechanism for this is that Y sperm become capable of fertilization before X sperm. The short co-incubation time would only allow sperm that were able to fertilize the oocytes quickly to produce embryos, while extending co-incubation would give the slower X sperm the time to fertilize oocytes.

Other published data suggested that there are differences between X and Y sperm in functional survival or capacitation rates. *In vitro* matured oocytes were

fertilized immediately after the first PB was extruded (immature) or 8 h later (mature). Immature oocytes produced more female embryos while mature oocytes produced more male embryos (Dominko and First, 1997, Gutierrez-Adan, et al., 1999). This suggests that Y sperm are able to fertilize oocytes before X sperm and that X sperm survive or remain fertile longer than Y sperm. Y sperm would be favored when sperm are exposed to oocytes that are ready to be fertilized because they would become capable of fertilization first. When sperm are added to oocytes that are immature, they must wait for the oocyte to become receptive to sperm. In this time the Y sperm lose the ability to fertilize the oocyte and the X sperm are favored because they survive longer.

One finding we did not expect was the lack of a difference in percent Y sperm from 2 to 12 h. The above studies found differences at earlier times (6-8 h), however these studies were conducted in the absence of oviductal cells. Co-incubating sperm with oviductal cells has been shown to delay capacitation and preserve sperm fertility (Pollard, et al., 1991). The delayed capacitation and preservation of the sperm by the oviductal cells could have increased the co-incubation time required to detect differences between X and Y sperm, which could explain why we did not see differences until after 12 h of co-incubation.

The percent Y sperm decreased significantly from 12 h to 24 h of *in vitro* co-incubation. In other words, a greater proportion of X sperm were attached to the oviductal cells at 24 h compared to at 12 h. This indicates that between 12 and 24 h more Y sperm became detached from the oviductal cells than X sperm. Thus, the X sperm had a greater ability to remain attached to or a stranger

attachment to the oviductal cells over time. This could explain the mechanism that causes an increase in the time between insemination and ovulation to favor production of female calves.

Binding to oviductal cells preserves sperm fertility (Pollard, et al., 1991). During the preovulatory period sperm remain in the oviductal reservoir (Hunter and Wilmut, 1984). Sperm are released from the oviductal reservoir after undergoing capacitation induced by changes in the oviductal secretions that occur around the time of ovulation (Parrish, et al., 1989b, Grippo, et al., 1995, Talevi and Gualtieri, 2001, Therien, et al., 2001). The timing of the changes in the oviductal fluid that induce capacitation are timed to release sperm at an appropriate time relative to ovulation to allow the sperm to fertilize the oocyte (Parrish, et al., 1989b, Grippo, et al., 1995, Talevi and Gualtieri, 2001, Therien, et al., 2001) However, sperm that become capacitated and released from the reservoir in the absence of these changes would be capacitated too far in advance of ovulation and would not be capable of fertilizing the oocyte once it is ovulated because capacitation decreases the life span of the sperm (Soupart and Orgebin-Crist, 1966, Bedford, 1970). In the experiments described here, the sperm that became detached during incubation represent sperm that released from the oviductal cells in the absence of any capacitation factors and the sperm that remained attached represent the sperm that would be part of the fertilizing pool sequestered in the oviductal reservoir.

When the time from insemination to ovulation is increased, the amount of time sperm spend in the oviductal reservoir is also increased. The fertilizing pool

of sperm will be the sperm that remain attached to the cells in the oviductal reservoir until the appropriate moment prior to ovulation when capacitation is triggered by changes in the oviductal fluid. Sperm that become detached during the pre-ovulatory period prior to this time are not likely to remain viable until ovulation occurs. Thus, if more X sperm than Y sperm were able to remain attached to oviductal cells over time, there would be more X sperm in the fertilizing pool. An increase in the proportion of viable X sperm in the fertilizing pool could increase the proportion of females conceived.

VI. RECOMMENDATIONS FOR FUTURE RESEARCH

As stated above, there is some evidence that X and Y sperm differ in ability to maintain fertilizing capacity over time. We have demonstrated that X and Y sperm differ in their ability to remain attached to oviductal cells over a period of time. The numerical increase in percent Y sperm from 24 to 36 h may have been found to be significant if there had been more statistical power. It is also possible that the percent Y sperm would increase further if the co-incubation time were extended. Further studies using longer co-incubation times are needed to investigate this possibility.

In vivo, sperm are not immediately exposed to oviductal cells. It can take 6 to 12 h for sperm to reach the oviducts and form a reservoir (Dobrowolski and Hafez, 1970, Hunter and Wilmut, 1984, Wilmut and Hunter, 1984). Experiments that more closely mimic the *in vivo* environment could be conducted by incubating sperm *in vitro* for different amounts of time prior to addition to

oviductal cells. Pre-incubation of sperm may also effect the ability of X and Y sperm to attach to the oviductal cells. Only uncapacitated, acrosome intact, morphologically normal sperm can attach to oviductal epithelium (Gualtieri and Talevi, 2000, Petrunkina, et al., 2001, Topfer-Petersen, et al., 2002, Gualtieri and Talevi, 2003). Previous research indicates that Y sperm become capacitated before X sperm (Dominko and First, 1997, Gutierrez-Adan, et al., 1999, Kochhar, et al., 2003, Lechniak, et al., 2003). Thus, aging sperm prior to co-incubation would be expected to decrease the ability of Y sperm to attach to the oviductal cells.

The decrease in the percent Y sperm from 12 to 24 h indicates more Y sperm became detached from the oviductal cells than X sperm during this time. Sperm are released from the oviductal reservoir *in vivo* once they undergo capacitation. The differences in the release of X and Y sperm from the cultured oviductal are possible due to differences in capacitation. However, capacitation was not studied directly. Studies comparing capacitation of X and Y sperm over time and in various conditions would be interesting and may uncover differences between X and Y sperm.

The complex *in vivo* environment of the female reproductive tract is difficult to duplicate in a laboratory setting. However, *in vivo* experiments can be expensive and difficult to perform. Our *in vitro* evidence of differences between X and Y sperm could justify experiments to determine if these differences exist *in vivo*. One option for *in vivo* experiments would be to remove the oviducts from cows after different times following insemination and collect the sperm that is and

is not attached to the oviductal cells. The proportion of X and Y sperm for attached and viable and unviable unattached sperm could be determined. This would allow the comparison of sperm that remained attached to the oviductal cells in the oviductal reservoir for different lengths of time.

VII. CONCLUSION

The research presented in this chapter indicated, for the first time, a difference between X and Y sperm in their ability to remain attached to oviductal cells over time. This work has provided a starting point for further studies aimed at investigating differences in X and Y sperm in their relation with oviductal cells both *in vitro* and *in vivo*.

CHAPTER FOUR

Obstacles and Solutions

I. REAL-TIME PCR ASSAY

A reliable, high throughput method to determine the proportions of X and Y sperm in samples was required to test our hypothesis. Because we could not find a suitable method reported in the literature, we developed a quantitative real-time PCR based assay to determine the proportion of Y sperm in samples. It was determined that two primer sets would be needed. One set would amplify both male and female DNA equally for estimation of total numbers of sperm. The other set would amplify only male DNA to determine the number of Y sperm in the sample. While it would be possible, we did not develop primers to determine the number of X sperm in the sample. This was not pursued for two reasons. First, the number of X sperm can be inferred based on the number of Y sperm and the total sperm. Second, it is easier to test Y specific primers for nonspecific amplification because female DNA, which does not have a Y chromosome, can be used. It is more difficult to test X specific primers for non-specific amplification because there is no available DNA that lacks an X chromosome.

Real-time PCR primers were determined using the following method: traditional PCR primers that had been published for sexing bovine embryos were used to amplify male DNA. One set of primers was male specific and the other amplified both male and female DNA. The PCR products were cloned into vectors and grown in *E. coli*, the product was sequenced and the plasmids were

used to create standard curves. The Primer Express program (Applied Biosystems) was used to determine the best real-time PCR primers within the sequence. These methods worked well to develop primers specific to the 1.715 satellite region for estimating the total number of sperm. However, several problems occurred during the development of the Y specific primers that made the development of the assay more difficult and time-consuming than expected.

The first male-specific traditional PCR primer set amplified a similar sized product when used on either male or female DNA. This was unexpected because these primers had been reported to sex embryos with 100% accuracy (Machaty, et al., 1993). This suggested that the sequence was present in both male and female DNA and therefore primers designed to amplify a portion of this sequence may not have been male-specific. A second set of male-specific traditional PCR primers was specific to male DNA and did not amplify female DNA, however the sequence yielded no usable real-time PCR primers.

At this point, we decided to change our approach because the published traditional PCR primers were not yielding usable results. Traditional PCR primers were designed to amplify two different sections a known sequence of a portion of the Y chromosome. The two sequences yielded four different real-time PCR primer sets; all gave non-specific amplification of female DNA. While our attempts to develop male-specific real-time PCR primers had failed thus far, we did find published real-time PCR primers that were reported to be male-specific (Joerg, et al., 2004). The published primers amplified a section of the SRY gene and the sequence around which the primers were designed was also reported.

We designed traditional PCR primers to amplify the published sequence of the SRY gene and verified that the sequence of our traditional PCR product matched the published sequence. We also verified that the real-time primers reported to be male specific did not amplify female DNA.

Standard curves were developed from the plasmids containing the cloned fragment of the SRY gene. Unfortunately the plasmids that contained the 1.715 satellite sequence were lost when a freezer broke and samples had to be moved. This could have been avoided had more care been taken during the rearrangement or had back-ups been made. New plasmids were made to replace those that were lost, but the sequence of the new plasmid did not match the original plasmid from which the 1.715 real-time PCR primers had been designed. The new sequence was off by one bp in the region where the forward primer was to anneal. This difference may not have affected amplification by the primers but we ordered new primers with the correct sequence for the new plasmids because we wanted to be as accurate as possible.

At the time of development we did not realize that the 1.715 satellite real-time PCR primers amplified a multi-copy sequence while the SRY real-time PCR primers amplified a single copy gene. In retrospect, designing both primers to amplify singe copy sequences may have improved the method and would be recommended if the assay were to be used for commercial purposes. For our purpose, we were able to use the assay as it was with some minor adjustments. First we used different dilutions of the plasmids to make the standard curves. Also, we used a different sample concentration with the different primers in order

to ensure the samples would appear within the standard curve of each primer. In addition a standard pool of male DNA of a known concentration was included in every real-time PCR run. This standard pool was used to calculate the within and between assay CVs and to calculate the number of Y sperm or total sperm in each sample.

The inter- and intra- assay CVs were acceptable during the validation of the assay as reported in Chapter Two. However, these CVs were based on a small number of samples because we did not have the extensive data that we obtained after the experiments of Chapter Three. Once these experiments were completed we found the inter-assay CVs to be unacceptably high. This was due to the method for making the standards dilutions of the plasmids used for the standard curve. The standards were made in small batches, enough for about six assays, and kept together. Because the standards were not divided into aliquots, they experienced a freeze-thaw cycle every time they were used. This freeze-thaw cycle caused the plasmids to become degraded so the actual number being amplified was lower in the later runs compared to the earlier runs of each batch, which caused the standard curve to shift down. This could have been avoided by making a large batch of standards, enough for all the assays, and dividing it into aliquots so that all the standards would have been uniform and would not have experienced repeated freeze-thaw cycles. We could not repeat all the assays due to time and financial constraints so this problem was corrected by excluding the assays that had inter-assay CVs greater than 20% from the data analysis.

II. SPERM-OVIDUCT CO-INCUBATION

We developed the methods used for sperm-oviduct co-incubation based on previously published methods because we had no prior experience in this area. Several preliminary experiments were conducted to determine the best methods for our purposes. Although there is evidence that the sperm binding sites on oviductal cells do not change with the hormonal stage of the cow (Lefebvre, et al., 1995, Suarez, 1998, Suarez, 2002), we decided to collect only oviducts from reproductive tracts that had no functional corpus luteum and at least one large follicle as was common in the literature. Collection and culture of the oviductal epithelial cells was relatively easy, except for a few times when cells had to be discarded due to contamination or cell death. Fresh cells from new collections easily replaced the cells that had to be discarded. Determining the details of the sperm-oviduct co-incubation was more difficult and time consuming.

We had to determine the ideal sperm suspension media to use during co-incubation. While sperm-TALP has been used successfully in previously published methods, these methods did not attempt to co-incubate the sperm for the extended period of time we were using. During some of the preliminary experiments the oviductal cells did not survive to the end of co-incubation. We felt this was due to the inability of sperm-TALP to support the oviductal cells over extended periods of time. IVF media was found to maintain the oviductal cells during the co-incubation and the cells incubated in IVF media appeared similar to cells incubated in culture media.

After the addition of sperm to the oviductal cells we wanted to remove the sperm that did not attach to the oviductal cells (unattached sperm). This ensured that sperm that were not attached to the oviductal cells at the end of each incubation time had once been attached and represented the sperm that became detached during the incubation. Preliminary experiments yielded the lowest number of unattached sperm when unattached sperm were removed after 2h of co-incubation. The result of this experiment is shown in Chapter Three.

After incubation we wanted to separate the live detached sperm from the dead detached sperm. A common method for separating live and dead sperm is centrifugation on a Percoll gradient. The live sperm collect in a pellet in the bottom of the centrifuge tube and dead sperm remain on the top of the Percoll layers. We compared two different Percoll gradients; a two layer Percoll, the layers of which consisted of 45% and 90% Percoll and a three layer Percoll, the layers of which consisted of 30%, 60%, and 90% Percoll. We found both Percoll gradients increased the percent live sperm in the sperm pellet and the three-layer Percoll gradient increased the percent live to a greater extent than the two-layer gradient. Although 100% live sperm was not observed, the timing of the stain could have been a factor, we cannot know if the sperm that stained dead were live after the Percoll and died during the staining or visualization processes. While 100% live/dead separation would have been preferred, we determined the Percoll gradient was the best available separation technique.

The next question we encountered was how to remove the sperm attached to the oviductal cells at the end of the co-incubation. Previously

published methods used heparin to remove sperm from oviductal cells, but heparin concentration and incubation time varied among experiments. We tested different heparin concentrations and incubation times to determine the optimal strategy for removal of sperm. We also tested different methods to help physically remove the sperm from the cells. The best sperm removal method was incubation in 200 μg/ml of heparin for 30 minutes at 38.5°C followed by washing five times by squirting the media gently onto the cells in a sweeping motion. Although this method was able to remove over 99% of sperm in preliminary experiments, many sperm remained attached to the oviductal cells during the first replicate of the extended co-incubation experiment. To address this, D-PBS was used to wash the cells an additional five times; this additional wash was able to remove most of the sperm from the oviductal cells.

Once sperm were collected, their DNA needed to be extracted for use in the real-time PCR reactions. At first we attempted to use the commercially available microLYSIS (Microzone Ltd; West Sussex, UK), however, this kit was designed for somatic cell lysis and was not able to lyse the highly condensed sperm nuclei. Another concern with the DNA extraction was that we did not want DNA from the oviductal cells to contaminate the samples. The highly condensed state of sperm DNA allowed us to remove somatic cell DNA that may have contaminated the samples. A previously published method was used to eliminate any contaminating oviductal cell DNA (Gill, et al., 1985). First an Epithelial Lysis mixture was added to the samples, this mixture was able to lyse somatic cells while leaving the highly condensed sperm nuclei unaffected. Any contaminating

DNA was easily washed away after centrifugation, without disturbing the sperm pellet. The stronger Sperm Lysis mixture that contained DTT was added to lyse the sperm nuclei and the sperm DNA was collected. Experiments were designed to determine the optimal volume and incubation time for the Epithelial Lysis mixture to eliminated oviductal DNA without reducing sperm DNA yield. Once the sperm nuclei were lysed, the DNA needed to be collected. The use of isopropanol to collect the sperm DNA after lysis did not yield consistent results. A P:C:I extraction procedure improved sperm DNA yield. After the method of sperm DNA extraction was established it was determined that a minimum of 100,000 sperm per sample would be required for use in the real-time PCR assay.

In order to ensure that each sperm sample contained enough DNA for use in the real-time PCR assay we tested different amounts of starting sperm. When one million sperm were placed in each well the number of live and dead detached sperm was insufficient; two million starting sperm per well were found to be optimal. It was determined that 12 wells per time-point was the maximum that could be completed; twelve wells of one 24-well plate were used for each time-point.

We planned to use sperm from six bulls so that each bull could be represented in two wells of each plate and we wanted to replicate the experiment four times so that we would have eight observations for each bull at each time-point. Because sperm from eight bulls was donated (Alta Genetics; Alberta, Canada), we altered sperm placement and replication. We considered using only six of the eight bulls donated, but we would not have had enough sperm to

complete all four replicates. Sperm from each bull was placed into six separate wells on each plate and four of the bulls were repeated in the four remaining wells. The bull placement was determined beforehand to ensure each bull had an equal number of wells for each time-point throughout all four replicates and placement varied to avoid positional biases. This allowed for each bull to have a total of 6 observations per time-point.

The experimental design required a minimum of twelve million sperm per bull. Two of the eight total straws per bull were thawed for each replicate. Unfortunately, there was not always enough live, motile sperm for each bull. During the first replicate three of the bulls had less than twelve million live motile sperm after separation on a Percoll gradient. The sperm suspension was adjusted to a total volume of 3 ml and all live motile sperm were used for the bulls that had twelve million or less. For the three bulls that had more than twelve million sperm, the concentration of the sperm suspension was adjusted to four million sperm per ml. Each well received between 1.42 and 2 million sperm, depending on the bull. In the second replicate, five bulls had less than twelve million sperm; I decided that all available sperm would be used for the remainder of the replicates. For the second replicate the wells received between 1.17 and 2.42 million sperm. The third and fourth replicates yielded more sperm per bull, with only two bulls having less than twelve million. In retrospect, it would have been better to continue as I had in the first replicate; using all available sperm when the amount was less than two million per well and reducing the amount to two million per well when it was more. This would have reduced the variation in

sperm per well. I didn't expect the increased number of sperm in the third and fourth replicates based on what I had in the first two replicates or I would not have changed the procedure. The number of starting sperm ranged from 1.17-4.98 million sperm per well for all bulls analyzed with an average of 2.37 million sperm per well. The number of starting sperm was included in the data analysis and was found not to have an effect on the results.

Bull four had low sperm numbers at all times and the sperm often looked unhealthy or dead. In addition, the oviductal cells that were incubated with sperm from bull 4 often died. Upon close examination, bacteria were found in the wells of bull 4 after longer incubation times. We believe the semen from bull 4 was the source of this contamination because bacteria were only found in wells with sperm from bull 4. One possible explanation for this is that the extender used for bull 4 was contaminated. Bull 4 was excluded from the statistical analysis.

Although we had planned on determining the amount of Y sperm in the live and dead detached sperm samples, there was not enough DNA to run the assay for these samples. This was unexpected because we increased the number of sperm used based on preliminary experiments to ensure there would be enough sperm in these samples. While we did not count the sperm in any samples before extracting the DNA, there should have been enough sperm for analysis. One possible explanation for this is that the detached sperm could have been damaged by oxidative stress during the incubation, causing the DNA to de-condense. This would have caused the de-condensed DNA to be discarded with the oviductal cell DNA, which could have reduced the total DNA yield to below

what was required for the analysis. Supporting evidence in the literature shows that prolonged incubation of sperm causes the DNA to de-condense due to free radicals in the storage media (Ferrandi, et al., 1992, Vishwanath and Shannon, 1997).

Another variable we should have determined was the percent Y of the sperm samples before addition to the oviductal cells. We did collect pre-incubation control samples before the Percoll separation for replicates three and four, but we should have collected samples of sperm after the Percoll separation for all the replicates to determine the starting percent Y sperm. This would have allowed us be sure that initial sperm percent Y sperm did not affect the results. Because the sperm for each bull was taken from one ejaculate, there should not be a large variation between replicates so the information we do have was useful. Ideally we would have had plenty of sperm for each bull and had taken a sample before running the sperm on the Percoll gradient, then diluted the live motile sperm to contain 4 million sperm per ml with an extra 0.5 ml for the control sample, so that the control sperm would have been the same as the sperm that were added to each well.

In retrospect, many of the aspects of these experiments could have been improved. The experience of making mistakes during the design and execution of the experiments has probably taught me more than if everything had been done perfectly the first time. Designing the methods myself gave me the opportunity to, not only learn laboratory techniques, but critical thinking skills that will be invaluable throughout my career.

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