To the Graduate Council:

I am submitting herewith a thesis written by Jennifer Miller entitled "Aging may reveal biological differences between X- and Y-bearing sperm." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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AGING MAY REVEAL BIOLOGICAL DIFFERENCES BETWEEN X- AND Y-BEARING SPERM

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much, more than I can remember. He will forever remain a part of me, reminding me just how truly amazing animals, especially horses, can be.

ABSTRACT

Overall objective of this thesis was to use aging as a way of investigating potential biological differences between X- and Y-bearing sperm. Specific objectives included the following: 1) examine effects of aging sperm 19.5 h post-thaw in an incubator at 38.5°C by evaluating embryonic development after fertilization; 2) examine effects of aging sperm within an artificial insemination (AI) straw for 8 and 14 h post-thaw in a water bath at 34.4°C or 23 h post-thaw in a refrigerator at 4°C by evaluating sperm motility and embryonic development after fertilization; 3) investigate effects of aging sperm within an AI straw 14 h post-thaw in a water bath or 23 h in a refrigerator for altering sex ratio of embryos after fertilization; and 4) examine effects of aging sperm within an AI straw 8 and 14 h post-thaw in a water bath for altering sex ratio of embryos after fertilization. Fertilization of oocytes with sperm aged 19.5 h in an incubator reduced proportion of putative zygotes (PZ) cleaved ($P<0.05$). However, ability of cleaved embryos to develop to 8-16 cell ($P>0.1$) or blastocyst was not compromised ($P>0.1$). Aging sperm in an AI straw 14 h post-thaw in a water bath or 23 h post-thaw in a refrigerator reduced proportion of motile sperm ($P<0.0001$). Moreover, fertilization of oocytes with sperm aged in an AI straw 14 h post-thaw in a water bath reduced proportion of PZ which cleaved ($P<0.05$); however, ability of cleaved embryos to develop to 8-16 cell ($P>0.1$) and blastocyst was not compromised ($P>0.1$). Interestingly, fertilization of oocytes with sperm aged in

an AI straw 14 h post-thaw in a water bath altered sex ratio of embryos, resulting in more females ($P<0.05$). In contrast, fertilization of oocytes with sperm aged in an AI straw 23 h post-thaw in a refrigerator did not alter sex ratio. Surprisingly, fertilization of oocytes with sperm aged in an AI straw only 8 h post-thaw in a water bath did not alter sex ratio of embryos. Blastomere numbers in male and female embryos did not differ ($P>0.2$). Results suggest the possibility that biological differences may exist between X- and Y-bearing sperm. If results are shown to be repeatable, such differences between X- and Y-bearing sperm may be exploited for the development of alternative methods of sex selection.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	REVIEW OF THE LITERATURE	3
	Benefits of Sex Selection	3
	Current Methodologies Used For Sex Determination and/or Selection	4
	Sexed sperm using flow cytometry	5
	Sex determination using polymerase chain reaction of embryo biopsies	7
	Sex determination using ultrasonography	9
	Spermatogenesis	11
	Fertilization	15
	Fertility lifespan of sperm	17
	Established and Inferred Biological Differences Between X- and Y-Bearing Sperm	18
	Potential differences in motility of X-and Y-bearing sperm	19
	Potential differences in density of X- and Y-bearing sperm	20
	Potential differences of X- and Y-bearing sperm in differing pH levels	21
	Potential differences in surface composition and charge between X- and Y-bearing sperm	21
	Potential differences in gene expression of X- and Y-bearing sperm	22

TABLE OF CONTENTS

Chapter		Page
	Altered Sex Ratios Suggesting Biological Differences Between X- and Y-Bearing Sperm May Exist	25
	Time of insemination may influence sex ratio	25
	Use of aged sperm may alter sex ratio of resulting embryos	30
	Potentially deleterious effects of aging sperm	31
	Summary and Experimental Objectives	33
III.	MATERIALS AND METHODS	34
	Materials	34
	In Vitro Maturation, Fertilization, and Culture of Bovine Embryos	35
	Evaluation of Sperm Motility	35
	Sexing of Cleavage Stage Embryos Using PCR Amplification	36
	Developmental Competence of Oocytes Fertilized With Sperm Aged in an Incubator 19.5 h	37
	Developmental Competence of Oocytes Fertilized With Sperm Aged 8 or 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C	38
	Sex Ratio of Embryos Produced Using Sperm Aged 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C	38
	Sex Ratio of Embryos Produced Using Sperm Aged 8 or 14 h in a Water Bath at 34.4°C	39
	Statistical Analysis	40

TABLE OF CONTENTS

Chapter	Page
IV. RESULTS	41
Developmental Competence of Oocytes Fertilized With Sperm Aged in an Incubator 19.5 h	41
Developmental Competence of Oocytes Fertilized With Sperm Aged 8 or 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C	42
Sex Ratio of Embryos Produced Using Sperm Aged 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C	44
Sex Ratio of Embryos Produced Using Sperm Aged 8 or 14 h in a Water Bath at 34.4°C	46
Effect of Aging on Motility of Sperm	48
Effect of Sex on Blastomere Number	48
V. DISCUSSION	50
Production of developmentally competent embryos from aged sperm	50
Effects of aging sperm post-thaw on motility of sperm	50
Effects of aging sperm post-thaw on embryonic development after fertilization	52
Fertilization of oocytes with aged sperm may alter sex ratio of embryos	55
Summary and implications	61
LITERATURE CITED	62
VITA	74

LIST OF TABLES

Table		Page
1.	Developmental potential of oocytes fertilized with control sperm or sperm aged 19.5 h before fertilization	41
2.	Progressive motility of control sperm and sperm that were aged 8 or 14 h in a water bath at 34.4 °C or 23 h in a refrigerator at 4°C	42
3.	Developmental potential of oocytes fertilized with control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C	43
4.	Development to blastocyst and number of nuclei for oocytes fertilized with control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C	43
5.	Developmental competence of oocytes fertilized with control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C	45
6.	Blastomere number comprising embryos produced using control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C	46
7.	Developmental competence of oocytes fertilized with control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C	47

LIST OF TABLES

Table		Page
8.	Blastomere number comprising embryos produced using control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C	48
9.	Effect of aging on proportion of motile sperm	49
10.	Effect of sex on blastomere number	49

LIST OF FIGURES

Figure		Page
1.	Electron micrograph of three type B rat spermatogonia joined by two intercellular bridges	13
2.	Electron micrograph of a pair of type A chinchilla spermatogonia connected by typical intercellular bridges	13
3.	Sex ratio of cleavage stage embryos produced using control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C	45
4.	Sex ratio of cleavage stage embryos produced using control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C	47

CHAPTER I

INTRODUCTION

Sex selection has intrigued the minds of many over the past several decades. The benefits of sex selection are vast and various for several aspects of the world, especially for the agriculture sector with its huge responsibility to supply the world an adequate food supply, as well as for humans in its use in the prevention of lethal sex linked diseases. The more efficient method of sex selection would be pre-fertilization in the selection of X- or Y-bearing sperm. Current methods of separating the two populations have been met with numerous limitations including complicated technical requirements, high cost, and poor efficiency. The development of a method to select X- or Y-bearing sperm that is efficient and readily usable by animal producers would be extremely advantageous. Development of an alternative method of sex selection at the level of sperm will be dependent upon identifying biological differences between X- and Y-bearing sperm. To date, no difference other than DNA content has been validated to exist. However, several studies suggest aging sperm may reveal biological differences. For example, Pursley and coworkers (1998) observed more female calves produced when dairy cows were inseminated early in the estrual period before ovulation. Sapp and Martin-DeLeon (1992) noted more male embryos when female mice were mated to males that had undergone various periods of sexual abstinence. The overall objective of this thesis was to

investigate the use of aging as a way of identifying potential biological differences in X- and Y-bearing sperm. Included were specific objectives to examine developmental competence of embryos produced from frozen-thawed aged sperm and the sex ratio of resulting embryos produced from frozen-thawed aged sperm.

CHAPTER II

REVIEW OF THE LITERATURE

For centuries, the idea of sex selection has resonated strongly with mankind spurring the generation of many theories on possible methods to manipulate it. Sex selection would be most beneficial to the agriculture sector in increasing genetic improvements, and to humans in helping to eliminate sex linked diseases. The following is a review of literature discussing the benefits associated with sex selection, current methodologies used to manipulate sex ratio and the drawbacks associated with those, as well as established and potential differences between X- and Y-bearing sperm that could serve to form the basis for developing a more efficient sex selection technique.

Benefits of Sex Selection

The ability to select the sex of an offspring could yield several potential benefits for the livestock industry as well as the human medical field. For example, the dairy industry could benefit greatly with the production of more females to replenish the milking herd and boost genetic progress by increasing the power of selection.

The beef and swine industries could benefit from sex selection as well. In the beef cattle industry, producers could benefit from sex selection through the production of more males to be used as feeder calves. Steers generally accrue fat more slowly than heifers (Berg and Walters, 1983) and exhibit higher gain to

feed ratios compared to heifers of similar weight (O'Mary, 1983). Seed stock operations could benefit from selection for females out of top producing cows nearing the end of their reproductive usefulness to provide replacement heifers, as well as selection for males to produce genetically superior bulls. With increased use of artificial insemination in the swine industry, sex selection would allow production of replacement females from top quality boars, which would greatly enhance genetic progress.

Humans could stand to gain substantially from sex selection. Today there are over 500 sex-linked diseases (Seidel and Johnson, 1999) that affect millions of people all over the world such as hemophilia, Duchenne muscular dystrophy, Lesch-Nyhan syndrome, and X-linked mental retardation. With the advent of sex selection, such diseases or afflictions could be greatly reduced if not eradicated. Sex selection could also benefit those couples seeking a child of a particular sex because of the social status associated with it in some cultures, or simply for family balancing.

Current Methodologies Used For Sex Determination and/or Selection

At least three sex selection techniques have been developed that are successful in selecting for male or female offspring. These techniques can be divided into the following two categories: pre-conception and post-conception (reviewed by Levin, 1987). A pre-conception technique places selection intensity on sperm and would be more efficient because efforts to pre-determine sex would occur before conception. Post-conception techniques place selection

intensity upon the embryo or fetus after sex has been determined at fertilization. Sex selection at this point is inefficient in that oocytes used to create embryos of the non-desired sex are wasted. Sexing sperm based on a difference in DNA content using flow cytometry is currently the only validated effective pre-conception technique (reviewed by Seidel, 1999). Embryo sexing using polymerase chain reaction (PCR) and ultrasonography are the only post-conception techniques available.

Sexed sperm using flow cytometry

Flow cytometry is a process whereby cells are separated based on some measurable or detectable difference existing between populations to be sexed (Fulwyler, 1965). Gledhill and coworkers were among the first to realize flow cytometry's potential for measuring DNA in individual spermatozoa to monitor mutagenic events caused by environmental radiation (reviewed by Johnson, 1994). In 1982, Pinkel and coworkers first performed sorting of sperm using flow cytometry for the sole purpose of isolating X- and Y-bearing sperm (reviewed by Johnson, 1994). Modification of a commercial flow sorting system to orientate, analyze and sort sperm of mammals based on DNA content was announced in 1986 (reviewed by Johnson, 1994). Improvements of the original system may allow for sexed sperm to become commercially available to the livestock industry within the next two years. Flow cytometry has been utilized to sex sperm for the purpose of producing embryos and live offspring in rabbits (Johnson et al., 1989),

pigs (Johnson, 1991), cattle (Cran et al., 1993, 1995), and humans (Levinson et al., 1995).

Separation of X- and Y-bearing sperm using flow cytometry is achieved by detecting and measuring differences in total DNA content which varies with species from 2.9% in humans to 12.5% in the creeping vole and a difference of 3.8% in the bovine (reviewed by Johnson, 1994). Differences in DNA content stem from a difference in size between the X and Y sex chromosomes. Axial chromatid length between X and Y sex chromosomes have been shown to differ by 6% in 24 species (reviewed by Johnson, 1994).

Sexing X- and Y-bearing sperm using a flow cytometer involves several steps (reviewed by Johnson, 1995b). Sperm to be sexed are first stained with a DNA specific dye, bisbenzimide, commonly known as Hoechst 33342. The difference in total DNA content between X- and Y-bearing sperm facilitates a slight difference in fluorescence intensity when exposed to ultraviolet (UV) light that can be detected using computer analysis. Droplets containing sperm are charged accordingly and deflected into corresponding pools by voltage plates.

Sexing mammalian sperm using flow cytometry (reviewed by Seidel, 1999) requires proper orientation of sperm because flatness of the head causes UV light to be emitted more intensely at the edges. With the advent of high speed sorting utilizing HiSON (high-speed sorter with orienting nozzle), percent of sperm oriented correctly increased from 25% to 70% (Johnson and Welch, 1999). Under routine sexing conditions using HiSON, a final yield of 20 to 30%

of total sperm before sorting is achieved, assuming 80% motility rate initially (Johnson and Welch, 1999). Discarded sperm are either dead, non-motile, aggregated, incorrectly oriented, or undeterminable due to staining aberrations.

Sexing sperm using flow cytometry may not be ideal for use in artificial insemination in livestock species (reviewed by Seidel, 1999). However, it has been used in human in vitro fertilization programs (Johnson et al., 1993). Sexing rates of 12 million sperm/hour (Johnson and Welch, 1999) limit the use of sexed sperm for artificial insemination in species such as swine (require 3 billion sperm per insemination; Johnson, 2000) and cattle (require 20 million sperm per insemination; Cran and Johnson, 1996). Cryopreservation could extend the use of sexed sperm. However, Schenk and coworkers (1999) reported lower motility rates and increased proportion of missing acrosomes for cryopreserved sexed sperm, although they suggested that cryopreservation may be more damaging than sexing. Potentially harmful repercussions of fertilizing with sperm sexed using flow cytometry include chromosomal abnormalities (Libbus et al., 1987), delayed embryonic development and increased rate of fetal degeneration (McNutt and Johnson, 1996). These studies suggest further improvements of the sexing process are needed.

Sex determination using polymerase chain reaction of embryo biopsies

Sex selection can be performed utilizing PCR of embryo biopsies (reviewed by Bredbacka, 1998). Biopsy is performed on early stage embryos, usually 6-10 cell stage in humans (Chong et al., 1993) and 16-cell as well as blastocyst stage

in cattle (reviewed by Bredbacka, 1998). The actual biopsy can be performed using a microblade (the most common method) or by removing individual blastomeres. Embryos of the desired sex, as determined by PCR, can be transferred into surrogate recipients to yield desired offspring.

Sex determination is performed through amplification of a specific region unique to the X or Y chromosome. Several regions unique to the X or Y chromosomes can be chosen for amplification (Zfx/Zfy; Kirkpatrick and Monson, 1993; Bredbacka et al., 1995; testis and ovary specific genes such as pYMT2/B and ZP3, respectively; Cui et al., 1993; and testis specific genes such as SRY; Cui et al., 1994). Regions chosen to amplify that are unique to the X chromosome or autosomal chromosomes provide a control to ascertain whether co-amplification of the sex determining regions occurred correctly. When both a gene on the Y chromosome and an autosome were amplified, an efficiency rate as high as 95% and an accuracy rate of 98% or higher was achieved (Thibier and Nibart, 1995). Bredbacka and coworkers (1995) reported 100% accuracy of sex determination with only Y chromosome amplification. Embryos that have undergone biopsy have been reported to establish pregnancy (reviewed by Thibier and Nibart, 1995; Bredbacka, 1998) and develop normally following transfer into recipients (Cui et al., 1993; Makondo et al., 1997; Shea, 1998).

Sex determination using PCR of embryo biopsies is technically demanding and time consuming as well as potentially harmful to the embryo if biopsy is performed incorrectly (reviewed by Bredbacka, 1998). The equipment necessary

to perform biopsy and PCR is costly and requires special considerations with regard to location, as cross contamination from foreign DNA is a major concern.

Sex determination using ultrasonography

Ultrasonography (reviewed by Rajamahendran et al., 1994) is performed using a probe that emits sound waves that are reflected back to differing degrees depending on what type of tissue is encountered. Fluid is non-echogenic producing a black picture, whereas bone is echogenic producing a white picture and tissues of intermediate density yield images of a gray intensity.
Since its development, it has become a widely used diagnostic tool in both veterinary and human medical fields (reviewed by Rajamahendran et al., 1994).

Post-conception sex selection may be performed with the use of ultrasonography if used in conjunction with selective abortion to eliminate animals of the non-desired sex. Ultrasound can be used to determine sex of a fetus between days 60-100 of gestation in cattle (reviewed by Rajamahendran et al., 1994), 55-64 in horses (Curran and Ginther, 1991), and approximately 112 days in humans (Harrington et al, 1996). Use of ultrasound for sex determination is evaluated per rectum in cattle and trans-abdominally in humans. Structures such as the genital tubercle at days 55-64 of gestation (Curran and Ginther, 1991), scrotum at days 50-60 (Kahn, 1990), scrotal swelling at days 73-119 (Muller and Wittkowski, 1986), and penis and scrotum or labial folds at 140 days (Harrington et al., 1996) are commonly used to determine fetal sex.

The accuracy of determining fetal sex using ultrasound is influenced greatly by the degree of training and experience of the technician. Muller and Wittkowski (1986) reported an accuracy rate of 94% between day 70 and 120 of gestation in the cow when using scrotal swelling as an indicator of sex. Curran and Ginther (1991) reported up to 100% accuracy between days 50 and 100 of gestation in the cow identifying the genital tubercle. In humans, observation of fetal genitalia proved over 96% correct (Harrington et al., 1996).

Although sex determination by way of ultrasound may be performed not long after conception, success often depends upon the technician's ability, position of the fetus, cooperation from the animal being scanned (Curran and Ginther, 1991; Curran, 1992), type of equipment, scanning frequency, and age and parity of the mother (Rajamahendran et al., 1994). Thus, the accuracy of sex determination by way of ultrasonography is highly variable. Selective abortion following sex determination using ultrasonography is an option in selection of sex, however highly inefficient. The amount of time and effort invested in establishing and maintaining a pregnancy for the length of time required to enable sex determination is of great consideration in deciding to terminate a pregnancy of a non-desired sex.

In reviewing the current methods commercially available for sex selection, it becomes apparent that the most efficient and/or practical method has not yet been devised. When considering sex selection through the use of sexed sperm, by way of flow cytometry, or PCR of embryo biopsies, the costs and technical

demands associated with these methods greatly diminish the practicality of their implementation into on-farm settings. The huge inefficiency associated with sex selection following sex determination by ultrasonography renders this technique virtually impractical except for extreme situations. Thus, the development of an alternative method to alter the sex ratio of offspring is in great demand.

An alternative method of sex selection must above all be efficient and thus should be a pre-conception technique. However, to develop a pre-conception sex selection technique at the level of sperm, one must have an understanding of spermatogenesis. Doing so will be critical in identifying possible biological differences between X- and Y-bearing sperm. Moreover, gaining an understanding of the demands placed upon sperm during the process of fertilization will be important in evaluating whether such a technique is feasible.

Spermatogenesis

Spermatogenesis is the process by which functional haploid spermatozoa are produced in the testes from diploid spermatogonia. It is reviewed in depth by several authors (Johnson, 1995a; Kerr, 1995; Escalier, 1999) and will be covered in brief in this section. Spermatogenesis is comprised of three major processes, spermatocytogenesis, meiosis, and spermiogenesis, all of which take place within the seminiferous tubule.

Spermatocytogenesis, as reviewed by Johnson (1995a), entails continuing mitotic divisions of spermatogonia to ensure a constant supply of germ cells that will ultimately serve to produce spermatozoa. In addition, at this step,

spermatogonia are diploid and can still yield both X- and Y-bearing spermatozoa. Spermatogonia are connected by intercellular bridges of cytoplasm (figures 1 and 2), which serve to synchronize development of spermatozoa and produce functionally equivalent X- and Y-bearing sperm through sharing of transcription products (reviewed by Grootegoed et al., 1995). Such connections remain intact until just prior to spermiation. The primary spermatocyte undergoes DNA replication and extensive RNA and protein synthesis prior to entering into meiosis (reviewed by Erickson et al., 1981).

Meiosis, as reviewed by Johnson (1995a), is the process whereby a diploid cell is reduced to four haploid cells. In spermatogenesis, meiosis begins when a primary spermatocyte enters into the first prophase, which is the longest step of meiosis. In fact, the primary spermatocyte, formed after the mitotic division of B spermatogonia, exhibits the longest lifespan of all germ cell types found in the germinal epithelium (Johnson, 1995a). During prophase 1 of meiosis, transcription of genes believed to be involved in chromosome pairing occurs (reviewed by Escalier, 1999). The X and Y chromosomes are contained within the sex body or vesicle and believed to be transcriptionally inactive during meiotic prophase (reviewed by Grootegoed et al., 1995). However, some studies suggest a reactivation of X-linked as well as Y-linked genes post-meiotically (Hendriksen et al., 1995; reviewed by Hendriksen, 1999). Differential gene expression of X- and Y-bearing sperm will be discussed in a later section.

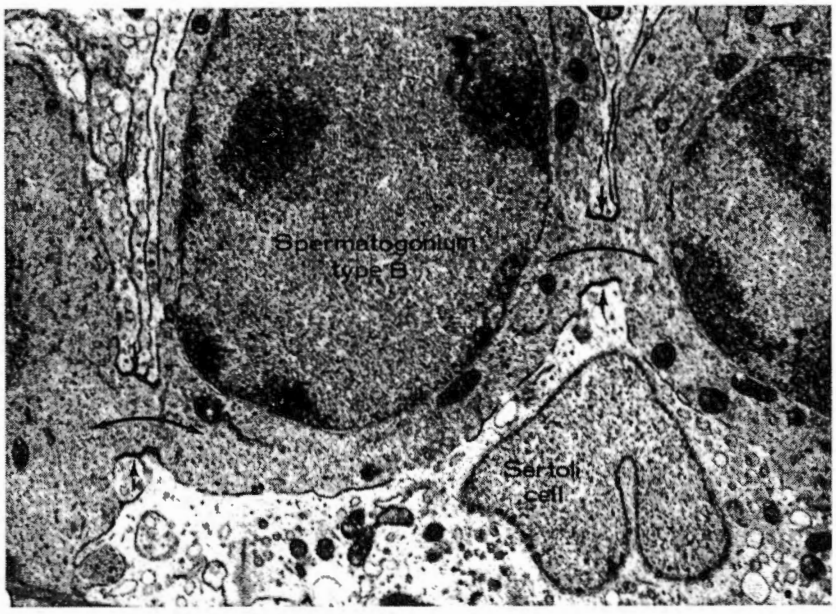


Figure 1: Electron micrograph of three type B rat spermatogonia joined by two intercellular bridges (Dym and Fawcett, 1971); arrows indicative of intercellular bridges.



Figure 2: Electron micrograph of a pair of type A chinchilla spermatogonia connected by typical intercellular bridges (Dym and Fawcett, 1971); arrows indicative of intercellular bridges.

The predecessors to X- and Y-bearing sperm, secondary spermatocytes, are formed during the first meiotic division when homologous chromosomes are separated. Secondary spermatocytes undergo a short interphase with no major DNA synthesis. The second meiotic division occurs rapidly to produce haploid round spermatids, which undergo morphologic changes during spermiogenesis to yield functional elongated spermatozoa.

Spermiogenesis, as reviewed by Johnson (1995a), entails an abundance of changes undergone by round spermatids to produce functional spermatozoa. The acrosome is produced by the Golgi apparatus, as are most enzyme-containing membrane systems. It contains digestive enzymes such as acrosin, hyaluronidase, and zona lysin whose main purpose is to aid in sperm entry into the zona pellucida of the oocyte. The spermatid nucleus elongates and the DNA undergoes extreme condensation. Chromatin histones change in arginine and cystine content along with the number of disulfide bonds. The flagellar canal is produced by an infolding of the plasma membrane and provides a mechanism by which new growth of the flagellum can be extended from the spermatid cell body. As the round spermatid begins to take on the appearance and makeup of a spermatozoon, excess cytoplasm is formed. This excess either remains with the seminiferous epithelium as residual bodies that are digested by Sertoli cells, or stays with shed spermatozoa as cytoplasmic droplets. During spermiogenesis, spermatids are connected by intercellular bridges, which are formed from incomplete membrane separation during cytokinesis. These spermatids are also

embedded in Sertoli cells, which act to provide material to stimulate growth and remove waste.

In the bovine, the entire spermatogenic cycle, as reviewed by Johnson (1995a), is approximately 61 days with a new wave of sperm being released every 13.5 days. In humans, spermatogenesis requires 74 days, with new sperm released every 16 days. Although sperm contain all the biochemical components necessary for fertilization, they still must undergo maturation in the epididymis of the male in order to successfully take part in fertilization. During maturation in the human epididymis, sperm acquire motility and loose the cytoplasmic droplet in preparation for fertilization (Bedford et al., 1973).

Fertilization

Fertilization is the complex process whereby the fusion of a spermatozoon and an oocyte produces a zygote genetically distinguishable from its parents that subsequently develops into the embryo that forms a live offspring at the end of gestation. It has been reviewed in depth by many authors (Ball et al., 1984; Dean, 1992; Barros et al., 1996; Sutofsky and Schatten, 1998; Wassarman, 1999a; Topfer-Peterson et al., 2000). Before fertilization, sperm and oocytes must undergo several modifications termed capacitation (reviewed by Fraser, 1992) and maturation (reviewed by Ball et al., 1984), respectively. The focus of this section, however, will be limited to events directly involved in fertilization.

Union of the oocyte and sperm occurs in the oviduct of the female reproductive tract. Binding of sperm to the zona pellucida (ZP) proceeds through

a receptor-ligand type binding (reviewed by Dean, 1992; Wassarman, 1999a; Wassarman, 1999b). The ZP is constructed of 3 major glycoproteins, ZP1, ZP2, and ZP3 (reviewed by Dean, 1992). Zona pellucida glycoprotein 1 and ZP2 are primarily structural while ZP3 acts as a sperm receptor in primary binding and contributes to induction of the acrosome reaction.

The acrosome reaction (reviewed by Barros et al., 1996; Wassarman, 1999a) begins with vesiculation of the acrosome membrane as it fuses with the sperm plasma membrane eventually releasing its contents. The sperm nucleus is exposed, surrounded by the inner acrosome membrane along with ligands such as fertilin. These ligands facilitate secondary binding of sperm to the oolemma, allowing the spermatozoon to be engulfed. To prevent polyspermy, structural changes occur in ZP3 that disrupt binding to additional sperm, termed the zona block (reviewed by Dean, 1992). These changes are thought to be a result of expulsion of cortical granules from the vitelline membrane of the oocyte during sperm entry. The vitelline membrane undergoes rapid depolarization to also block entry of additional sperm.

Following sperm entry into the oocyte, systematic dismantling of the spermatozoon takes place (reviewed by Sutovsky and Schatten, 1998). In mammalian fertilization, the sperm provide the centriole, released during such dismantling, that is necessary for microtubule organization during fertilization and first embryonic cleavage (reviewed by Sutovsky and Schatten, 1998). Breakdown of the sperm nuclear envelope exposes the genetic material

facilitating decondensation of the DNA and formation of the male pronucleus. Both the male and female pronuclei become apposed by the action of sperm aster microtubules. Following replication of DNA within both pronuclei, the chromatin recondenses and the zygote enters mitosis.

Fertilization performed in vitro exhibits slight differences from that performed in vivo in that sperm are not required to navigate the female reproductive tract. Instead they are placed directly in the oocytes' vicinity in much larger numbers than would be seen in the oviduct at fertilization (Suarez, 1987).

Fertility lifespan of sperm

The period of time in which sperm are able to retain the ability to fertilize an oocyte and produce a viable embryo is finite and varies according to species (reviewed by Austin, 1975). Sperm may remain fertile in the female reproductive tract, specifically the oviduct, for 24 to 48 hours in bovine and humans. In some bat species, sperm are fertile for up to 198 days.

Sperm may remain motile much longer, even though fertility may have already been compromised (reviewed by Austin, 1975). For example, bovine sperm may remain motile for up to 96 hours, whereas in humans sperm can retain motility up to 8 days. Manipulation of sperm, such as freezing, may shorten durations of motility and fertility, however, this may depend heavily upon the medium in which sperm are held. For example, frozen-thawed semen that was re-diluted for multiple inseminations was only fertile for a few hours (reviewed by Vishwanath and Shannon, 2000). However, with the advent of a

diluent called CAPROGEN®, re-diluted frozen-thawed sperm could be used efficiently for inseminations performed within one working day (reviewed by Vishwanath and Shannon, 2000). This method has since been improved to allow freezing of large volumes of semen (5-25 mL) for subsequent re-dilution and use for insemination within 60 hours of re-dilution (reviewed by Vishwanath and Shannon, 2000). Re-diluted semen used beyond 2 days reduced non-return rates.

With an understanding of spermatogenesis and fertilization, as well as the period during which sperm may remain both fertile and motile, one may proceed with development of a pre-conception sex selection technique involving the manipulation of sperm. Biological differences between X- and Y-bearing sperm must be identified to assist in developing such methods. Many biological differences have been inferred to exist, however, DNA content remains the only scientifically accepted difference (reviewed by Johnson, 1996).

Established and Inferred Biological Differences Between X- and Y-Bearing Sperm

Sex selection of offspring performed via selection of X- or Y-bearing sperm will be dependent upon identifying biological differences between the two populations. Such differences could be physical and/or biochemical. At present, the only biological difference scientifically validated is total DNA contained within X- and Y-bearing sperm (reviewed by Johnson, 1996). However, several studies have attempted to prove the existence of additional biological differences

(reviewed by Glass, 1976; Kovacs and Waldron, 1987; Levin, 1987; Zarutskie et al., 1989; Johnson, 1992; Windsor et al., 1993; Johnson, 1994; Johnson, 1995; Johnson, 1996; Reubinoff and Schenker, 1996; Steinbacher and Gilroy, 1996; Hossain et al., 1998; Seidel, 1999;).

Potential differences in motility of X- and Y-bearing sperm

The potential existence of differences in motility between X- and Y-bearing sperm has been investigated using different sperm purification techniques. For example, Ericsson and coworkers (1973), after isolation of higher proportions of Y-bearing sperm in albumin columns identified using quinacrine staining (stains section of Y chromosome), proposed that Y-bearing sperm swim faster and exhibit a greater ability to penetrate fluid interfaces. Others reported similar observations using quinacrine staining (Dmowski et al., 1979; Quinlivan et al., 1982) and sex ratio of offspring (Corson et al., 1984; Beernink et al., 1993) as their endpoints. However, reports of unsuccessful separation of X- and Y-bearing sperm using sperm DNA karyotyping (Brandriff et al., 1986) and fluorescence in situ hybridization (Flaherty et al., 1997) as their endpoints were also reported. Additional sperm purification techniques utilizing motility of sperm, swim-up and modified swim-up, were also reported to skew the sex ratio of offspring (Check and Katsoff, 1993; Khatamee et al., 1999). However, these results were in contrast to the findings of others using sex ratio of offspring (Check et al., 1994) and proportion of X- and Y-bearing sperm (Han et al., 1993; Lobel et al., 1993) as their endpoints. Differences in motility of X- and Y-bearing

sperm were investigated in a more direct manner using computer analysis by which it was determined that Y-bearing sperm did not swim faster than X-bearing sperm (Penfold et al., 1998). However, X- and Y-bearing sperm were shown to differ with regards to linearity and straightness of path, which could be due to post-meiotic modifications to the flagellum or other sperm factors. The authors were doubtful that differences in motility would provide a means of separating X- and Y-bearing sperm.

Potential differences in density of X- and Y-bearing sperm

On the supposition that X-bearing sperm are more dense than Y-bearing sperm, centrifugation of sperm through Percoll gradients was performed by Kaneko and coworkers (1983). Using quinacrine staining, authors reported an enrichment of 73% Y-bearing sperm in the lighter fraction. Using fluorescence in situ hybridization, Wang and coworkers (1994) observed a slight enrichment of X-bearing sperm (55.1%) in the densest layer of Percoll but commented that described differences were not enough for use in sex selection. However, Upreti and coworkers (1988) reported no enrichment of either X- or Y-bearing sperm after centrifuging through Percoll using flow cytometry to determine proportion of X- and Y-bearing sperm. Differences in density were also investigated using sedimentation (Schilling, 1966). When combined with centrifugation, sperm isolated through a medium of high viscosity resulted in a higher proportion of female calves. Luderer and coworkers (1982) attempted to separate X- and Y-bearing sperm based on density using Newtonian gels but were unsuccessful in

altering the sex ratio of 60 day-old bovine fetuses. It seems density does not differ sufficiently to allow successful separation of X- and Y-bearing sperm.

Potential differences of X- and Y-bearing sperm in differing pH levels

Among potential differences between X- and Y-bearing sperm is a differential ability to survive and retain motility in various pH conditions. Shettles (1970) proposed that acidic conditions favor X-bearing sperm and alkaline conditions favor Y-bearing sperm. However, this theory was refuted by results reporting no differential ability of sperm to survive various pH conditions when F-body identification using quinacrine staining (Downing and Black, 1976) and live offspring (Muehleis and Long, 1976) where used as endpoints. Thus, effects of pH on survivability of X- and Y-bearing sperm do not appear to be feasible for separation.

Potential differences in surface composition and charge between X- and Y-bearing sperm

Surface composition of X- and Y-bearing sperm, with respect to H-Y antigen and other abundantly expressed proteins, has been investigated by several researchers (reviewed by Hendriksen, 1999). Flow cytometry allowed examination of H-Y antibody binding to X- and Y-bearing sperm and showed similar binding for both populations. Flow cytometry also provided a means to examine protein composition on X- and Y-bearing sperm as well as different surface proteins. No differences were reported for bull and boar sperm (reviewed by Hendriksen, 1999). Engelmann and coworkers (1988) observed

separation of X- and Y-bearing sperm using free-flow electrophoresis; however, Manger and coworkers (1997) did not. Using ion-exchange column chromatography, Downing and coworkers (1976) also did not observe separation of X- and Y-bearing sperm. Thus, a difference in surface charge or protein composition within or on the surface of sperm does not appear to differ repeatedly for use in sex selection.

Potential differences in gene expression of X- and Y-bearing sperm

There are several studies to suggest biological differences between X- and Y-bearing sperm could be manifested through haploid gene expression and/or unequal sharing of translation products during spermatogenesis (reviewed in depth by Erickson et al., 1981; Lele and Wolgemuth, 1998). The primary spermatocyte, originating from B spermatogoina (reviewed by Johnson, 1995a), undergoes DNA replication and extensive RNA and protein synthesis prior to entering into meiosis (reviewed by Erickson et al., 1981). RNA synthesis was originally proposed to be minimal after meiosis; however, later studies showed only a 4-fold decrease during meiosis (reviewed by Erickson et al., 1981). Studies with mouse testicular cells showed high rates of RNA synthesis in post-meiotic cell stages with the highest level of [^{3}H] uridine incorporation in early spermatids (reviewed by Erickson et al., 1981). This new RNA may or may not be messenger RNA (mRNA) but studies suggest heterogeneous nuclear RNA is synthesized after meiosis (reviewed by Erickson et al., 1981). The X and Y chromosomes were believed to be transcriptionally inactive during meiotic

prophase; however, studies suggest a reactivation of X-linked as well as Y-linked genes post-meiotically (Hendriksen et al., 1995; reviewed by Hendriksen, 1999). Transcriptional elements may accumulate in early haploid spermatids to facilitate synthesis of mRNA needed for spermiogenesis (Lele and Wolgemuth, 1998).

Widely accepted is the notion of post-meiotic translation including several sperm-specific enzymes and antigens (reviewed by Erickson et al., 1981). Proteins specific to post-meiotic stages have been shown to exist (reviewed by Erickson et al., 1981). Non-Mendellian segregation of *t*-alleles, responsible for several severe mutations and may confer differential motility in sperm, may suggest the possibility of haploid gene expression (reviewed by Erickson et al., 1981). The gene product is thought to function at the cell surface and thus may be detected as a surface antigen. Surface antigens may be less likely to be transported through intercellular bridges and thus could result in unequal spermatids and therefore non-Mendellian segregation, commonly observed with the *t*-allele (reviewed by Erickson et al., 1981).

Evidence of differential development rates of male and female bovine embryos, prior to genome activation, may also support the theory of differential gene expression in X- and Y-bearing sperm. For example, Bredbacka and Bredbacka (1996) reported male embryos as having 1.25 more cells per embryo than females at 48 hours following insemination, but only in the presence of glucose. They attribute this difference to differential expression, in male and female embryos, of housekeeping genes glucose-6-phosphate dehydrogenase

(G6PDH) and hypoxanthine phosphoribosyl transferase (HPRT), which are potentially involved in controlling the levels of oxygen radicals, and thus oxidation-induced gene expression. They conclude that such housekeeping genes may be the first to be activated in the embryo, and thus may confer differences between male and female embryos prior to genome activation. However, it could also be argued that such differential gene expression originated during spermiogenesis through post-meiotic gene expression. Post-meiotic gene expression on the X chromosome has been demonstrated by Hendriksen (1995).

Differences in haploid gene expression would be expected to be minimal due to the presence of intercellular bridges between developing spermatids (similar to those shown in figures 1 and 2), however, consideration of unique membrane characteristics that help to maintain bridges may limit sharing of membrane components (reviewed by Erickson et al., 1981). Thus, the presence of surface antigens on sperm could demonstrate haploid expression (reviewed by Erickson et al., 1981). Mitochondria probably do not pass through intercellular bridges and thus gene products associated with them also may not be shared (reviewed by Erickson et al., 1981). Distribution of the sex chromosomes is assumed to be random and thus the chance that a spermatid is connected to one X- and one Y-bearing spermatid is 75%, while the chance of an X-bearing spermatid being connected to two other X-bearing spermatids is only 12.5% (the same would be true for Y-bearing spermatids; reviewed by Seidel, 1999). However, if

segregation is not random, there remains the possibility that several X- or Y-bearing sperm reside next to one another. Haploid gene expression may establish potential transcriptional gradients that could facilitate biological differences between the two sexes.

Altered Sex Ratios Suggesting Biological Differences Between X- and Y-Bearing Sperm May Exist

Time of insemination may influence sex ratio

Time of insemination as it relates to time of ovulation has been reported to affect sex ratio of resulting offspring. For example, Pursley and coworkers (1998) demonstrated an effect of time of insemination on sex ratio of calves in lactating dairy cattle. Estrus was synchronized in 732 lactating dairy cows using the following Ovsynch protocol: an injection of Gonadotropin Releasing Hormone (GnRH) was given followed seven days later by an injection of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) followed two days later with a second injection of GnRH. Ovulation was estimated to occur 24 to 32 hours following the second GnRH injection. Artificial insemination was performed once at 0, 8, 16, 24, or 32 hours post second GnRH injection. Sex of calves was noted at term. More female calves were observed from cows inseminated 0 hours post GnRH and again at 32 hours post GnRH (61 and 65% respectively). Percentage of female calves from cows bred at 8, 16, and 24 hours post GnRH were not different from the expected 1:1 (45, 54, and 54% respectively). The overall sex ratio of all calves produced was not different from 1:1. Wehner and coworkers (1997), using an OVATEC vaginal pH probe,

indirectly studied the effects of aging on sex ratio of calves. Estrus was synchronized in all cows using two $PGF_{2\alpha}$ injections 10 days apart and cows were inseminated at a declining reading of 45, corresponding to approximately 20 hours pre-ovulation, or a rising reading of 50-70, corresponding to approximately 8 hours pre-ovulation. Cows bred 20 hours pre-ovulation produced 1 (7%) male out of 14 calves as compared to those bred 8 hours pre-ovulation yielding 11 (92%) males out of 12 calves. Cows inseminated according to visual observation of standing estrus and those serviced naturally yielded 10 out of 20 (50%) and 16 out of 29 (55%) male, respectively. This study was limited with a small number of experimental units.

Effects of time of insemination have also been investigated in white-tail deer in which final copulation during estrus was evaluated with regards to sex ratio of fawns produced (Verme and Ozoga, 1981). Ovulation was estimated to occur 12-14 hours after termination of estrus. Matings consummated early in estrus (13-24 hours) resulted in only 4 (14.3%) males out of 28 fawns whereas matings later in estrus (49-96 hours) increased to 21 (80.8%) males out of 26 fawns. The overall sex ratio of all fawns produced, regardless of when in estrus conception occurred, was not different from 1:1.

Gutierrez-Adan and coworkers (1999) inseminated multiparous and nulliparous ewes at 0-6 hours prior to ovulation and 2-8 hours after ovulation. Sex ratio of lambs produced from multiparous ewes, all offspring included, was variable with more males produced at 6 hours before ovulation (93;

approximately 60%) as well as 2 hours after ovulation (90; approximately 60%). However, analysis of multiparous ewes with single offspring showed a higher proportion of males with inseminations 2 and 5 hours after ovulation (18 & 8, respectively; both approximately 85% male). All other insemination times were not different from 1:1. Insemination of nulliparous ewes before, at, or after ovulation did not yield sex ratios different from 1:1.

In humans, several retrospective and some prospective studies have been performed to investigate the effect of time of intercourse or insemination on the sex ratio of children. Although alterations are reported, the direction and magnitude vary according to study. France and coworkers (1984) reported, of 33 pregnancies, 68% of male children produced resulted from intercourse that occurred before ovulation and 64% of females at or after ovulation. In contrast, Harlap (1979) reported, of 3,658 births, more males (65.5%) were produced when intercourse occurred after ovulation. Some studies even compared the effects of natural copulation versus artificial insemination. Using information from 1,823 births, Guerrero (1970) reported opposite effects for natural and artificial with natural copulation yielding more males prior to ovulation but decreasing as ovulation approached, and artificial insemination yielding more females prior to ovulation and increasing as ovulation approached. Guerrero (1974) confirmed these findings in a second similar study.

In some studies, alterations in sex ratio of offspring were not affected by time of insemination. This was possibly due to differences in experimental design.

Soede and coworkers (2000) inseminated sows at various times before and after ovulation as determined by ultrasound (36-24, 24-12, and 12-0 hours prior to ovulation and 0-12 hours after ovulation). The percentage of male piglets were 52.1%, 50.5%, 54.9%, and 47.8% for sows inseminated during the 36-24, 24-12, and 12-0 hours before ovulation and the 0-12 hours after ovulation, respectively. Aging sperm in vivo did not affect sex ratio of piglets born. Additionally, time of insemination has also been shown not to affect sex ratio of offspring in cattle. Rorie and coworkers (1999) synchronized estrus in beef heifers and cows and monitored using a computerized HeatWatch system. Assuming ovulation occurred 28-32 hours after the onset of estrus, animals were artificially inseminated at 8-10 or 20-25 hours after onset of estrus. Ultrasound at 60-80 days post insemination was used to diagnose pregnancy and fetal sex. There was no effect of time of insemination on sex ratio of 68 total fetuses sexed (53.8% and 51.7% male for 8-10 and 20-25 hour groups, respectively). Ballinger (1970) also reported no effect of altered time of insemination on sex ratio of 1,000 calves produced from cows/heifers inseminated 0-12 hours into estrus (50% male) versus 12-24 hours (51% male).

Martin and Shaver (1972) delayed ovulation 14-31 hours in rabbits by injecting females with human chorionic gonadotropin 4-21 h after insemination. Blastocysts were recovered 6-7 days following ovulation and chromosome spreads were analyzed for sex chromosome complement. There was no effect of aging sperm on the sex ratio of blastocysts produced (46% (51) male for

control and 61% (80) male for all aged periods combined). Perhaps a larger experiment would have yielded significance between these groups. They also reported a higher occurrence of chromosomal abnormalities when sperm was aged (1% versus 9.7% for control and aged, respectively).

Effect of time of insemination on sex ratio is conflicting in humans as well. Gray and coworkers (1998) observed equal distribution of male and female children regardless of insemination before, at, or after ovulation. These results may be affected by their reliance on basal body temperature and mucous peak as predictors of ovulation.

Several plausible explanations may exist for reports of altered sex ratios after manipulating time of insemination. For example, sperm deposited after ovulation may result in aging of the oocyte. The oocyte may undergo changes within the maternal environment that ultimately select for one sex over the other. In addition, sperm deposited before ovulation may experience selective conditions as well. However, one could also speculate that sperm inseminated before ovulation may simply experience aging, which may result in increased fertilization by X-bearing sperm over Y-bearing. In the following section, effect of sperm age on sex ratio was investigated. However, aging was carried out in the male reproductive tract.

Use of aged sperm may alter sex ratio of resulting embryos

Sapp and Martin-DeLeon (1992) aged sperm in the reproductive tract of male mice by requiring them to undergo abstinence for 14 days after they were last

mated. Control males were prevented from mating for 3 days. Males were then allowed to copulate with females and 1-cell embryos at the first metaphase division were recovered approximately 33 hours post-mating. C-banding of chromosome preparations was used to determine X- or Y-bearing fertilization. X:Y ratio of 152 analyzable zygotes resulting from aged sperm was 1.53 in favor of males as compared to 0.94 for 171 analyzable zygotes from sperm not aged.

Effect of aging sperm in the male reproductive tract on sex ratio of embryos is contradictory as well (Sapp and Martin-DeLeon, 1992). In mice, sperm aged 14 days after the last mating in the male reproductive tract was used for in vitro fertilization of oocytes. Following 5-7 hours of incubation, embryos were collected and chromosome spreads were analyzed for sex chromosome complement. No effect of aging on sex ratio was observed (sex ratio of 1.01 for 157 analyzable embryos versus 0.97 for 169 analyzable embryos for control and aged, respectively). This lack of effect of aging could be attributed to the technique used to distinguish male from female zygotes. In this study, roughly half the embryos were analyzable.

Given the reports presented here, it remains unclear if aging sperm, by manipulating time of insemination or through male sexual abstinence, alters sex ratio. Results of studies are extremely conflicting possibly due to experimental size, technique for determining sex of embryos/fetuses, grouping of time intervals prior to and after ovulation, and determination of ovulation time. Further investigation into this phenomenon using in vitro aging of sperm to remove

effects of maternal or paternal environment may be greatly justified. Aging, however, can have detrimental effects on sperm, which may or may not lead to a reduced ability to fertilize and produce a developmentally competent embryo. Thus, perhaps the use of aging sperm should be limited to identification of biological differences between X- and Y-bearing sperm that could ultimately be used in developing alternative sex selection methods.

Potentially deleterious effects of aging sperm

Although aging sperm, whether in vivo or in vitro, may facilitate expression of potential biological differences between X- and Y-bearing sperm, the deleterious effects that aging for extended periods of time has on sperm are very serious. Aging gametes in general is thought to reduce fertilization rate and developmental competence of embryos formed. Fertilization of mouse ooyctes in vitro with sperm aged in vitro (0-6, 9-15 and 18-24 hours) reduced the ability of zygotes to reach prometaphase; however, aging sperm (0-6 and 9-15 hours) did not affect ability to penetrate oocytes and form pronuclei (Smith and Lodge, 1987). When in utero-aged (29-31 hours) sperm were used to inseminate rabbits, a decreased rate of fertilization, an increased rate of post-implantation loss, and reduced litter sizes were reported (Tesh, 1969). However aging (10-32 hours) did not affect pre-implantation loss, fetal weight, placental weight, or sex ratio. Martin-DeLeon and Boice (1982) reported an increase in chromosomal anomalies such as monosomy, trisomy, triploidy, and structural rearrangements when using sperm aged 6-20 days in the male reproductive tract. They also

reported a decrease in motility and fertilization rate associated with sperm aged in the male reproductive tract. Effects of storage on sperm at temperatures above 0°C have been reviewed by Vishwanath and Shannon (1997). Effects can be attributed to extracellular and intracellular factors. Extracellular factors include reactive oxygen species as well as factors found in seminal fluid, which upon extension and storage of sperm can have detrimental effects on sperm motility and fertility. Peroxide is the most detrimental of the pro-oxidants formed with the main source during ambient-temperature storage being oxidative deamination of aromatic amino acids by membrane-bound aromatic amino acid oxidase (AAAO). Activity of this enzyme has been shown to be restricted to dead sperm (reviewed by Vishwanath and Shannon, 1997). The large protein fractions in seminal plasma may disaggregate and damage sperm membranes (Vishwanath and Shannon, 1997). Intracellular events such as respiration and oxidative phosphorylation produce oxygen radicals, which can cause damage to mitochondrial membranes. With no repair or turnover mechanism in fully differentiated sperm, mitochondrial degeneration is detrimental to the function of sperm. Oxygen radicals can also damage the nuclear membrane affording access to DNA. However, the extreme condensed nature of sperm chromatin affords a relatively high degree of protection. Therefore, the potential effects of aging for extended periods of time, especially beyond the fertile lifespan of sperm, on fertility of sperm and developmental competence of embryos formed cannot be taken lightly. Thus, caution should be used when choosing aging

durations for sperm as a potential means of expressing biological differences between X- and Y-bearing sperm.

Summary and Experimental Objectives

Sex selection could provide many potential benefits for the livestock industry as well as humans. Commercially available sex selection techniques include both pre- and post-conception techniques. However, these techniques are limited by cost and the technical and laborious demands associated with them. Development of an alternative method of sex selection involving the manipulation of sperm requires identification of potential biological differences that may exist between X- and Y-bearing sperm. Aging sperm prior to fertilization may facilitate identification of such differences. The overall objective of this thesis was to use aging as a way of identifying potential biological differences between X- and Y-bearing sperm. Specific objectives to examine developmental competence of embryos produced from frozen-thawed aged sperm and the sex ratio of embryos produced from frozen-thawed aged sperm were included.

CHAPTER III

MATERIALS AND METHODS

Materials

All chemicals and reagents required for maturation, fertilization, and culture of bovine embryos, as well as Hoechst 33342, Dulbecco's phosphate buffered saline (DPBS), potassium sodium tartrate, eosin, nigrosin, polyvinyl alcohol, and pronase were purchased from Sigma (St. Louis, MO), unless otherwise noted. Frozen semen was generously provided by various companies and ovaries were purchased from Brown's Packing Company (Gaffney, SC). Medium-199 (M-199), gentamicin, penicillin-streptomycin, and non-essential amino acids (NEAA) were purchased from Specialty Media, Inc. (Phillipsburg, NJ). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Follitropin V was provided by Vetrepharm Canada, Inc. (London, Ontario) and Luteinizing hormone (LH) was provided by USDA (Beltsville, MD). Light mineral oil, giemsa, and permount were obtained from Fisher (Pittsburg, PA). Ampli-Y bovine embryo sexing PCR kits were generously provided by Dr. Peter Bredbacka (Finnzymes; Espoo, Finland). Modified Tyrode's media (HEPES-TALP, IVF-TALP, and Sperm-TALP; Parrish et al., 1988) and KSOM (Biggers et al., 2000 with modifications provided by Dr. John Hasler) were prepared in the laboratory or purchased from Specialty Media, Inc. (Phillipsburg, NJ).

In Vitro Maturation, Fertilization, and Culture of Bovine Embryos

Procedures utilized for in vitro production of embryos were as described by Edwards and Hansen (1996) with a few modifications. Briefly, cumulus oocyte complexes (COCs) were matured in M-199 containing Earle's salts, 10% FBS, 50.0 μg/mL gentamicin, 5 μg/mL FSH, 0.3 μg/mL LH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine (maturation medium) in a humidified atmosphere of 5.5% CO_2 and air. Oocytes were fertilized with Percoll-purified sperm [375,000 sperm/500 μL IVF-TALP including 0.5 mM penicillamine, 0.05 mM hypotaurine, and 10 μM epinephrine (PHE)] for 8-18 h at which time putative zygotes (PZ) were vortexed and washed extensively in HEPES-TALP to remove associated cumulus cells and spermatozoa. Putative zygotes were cultured in KSOM supplemented with non-essential amino acids for up to 9 days post-fertilization in a humidified atmosphere of 5.5% CO_2, 7% O_2, and 87.5% N_2. On day 4 post-fertilization, 8-16-cell embryos were transferred to fresh KSOM supplemented with non-essential and essential amino acids. Ability of putative zygotes to cleave and develop to blastocyst was assessed day 3 and 8-9, respectively.

Evaluation of Sperm Motility

Progressive motility of sperm was evaluated, as described by Sorensen (1979), with a few modifications. Briefly, sperm were diluted 1:20 in Sperm-TALP. Five microscope fields were evaluated in a 25 μL drop at 400X in which 10 random sperm per field were evaluated for progressive motility (movement in

a forward direction). The assessment was repeated, evaluating 100 sperm in total per semen sample.

Sexing of Cleavage Stage Embryos Using PCR Amplification

Determination of sex of cleavage stage embryos was conducted 43-68 hours post-fertilization using PCR kits generously provided by Dr. Peter Bredbacka. Primers were designed to amplify an undisclosed region on the Y chromosome. Embryos were subjected to 0.5% pronase to remove the ZP and subsequently processed through DPBS (without $CaCl_2$ or $MgCl_2$) followed by DPBS containing 0.09 mM $CaCl_2$, 0.49 $MgCl_2$, and 0.1% polyvinyl alcohol. Blastomeres were disassociated and counted before transfer to PCR tubes. Successful transfer of blastomeres to PCR tubes was confirmed by visual inspection of PCR tubes using a stereomicroscope. A lysis solution containing an undisclosed amount of Proteinase K was added to each tube. Lysis of blastomeres was performed at 55°C for 5 minutes followed by inactivation of Proteinase K at 95°C. Control tubes containing purified male and female DNA, included in kit, were placed in PCR machine with sample tubes. A PCR buffer containing Y-specific primers and ethidium bromide was added to all tubes, including controls. All samples were subjected to 10 cycles of 20 seconds at 94°C, 40 seconds at 50°C, 20 seconds at 75°C, followed by 35 cycles of 10 seconds at 96°C, 30 seconds at 60°C, 25 seconds at 70°C. Reaction products within tubes were then exposed to 312 nm UV light. Amplification of a Y chromsome-specific region resulted in only male embryos fluorescing due to the inclusion of ethidium bromide within the

reaction tubes. Lack of Y-specific sequences prevented amplification and thus fluorescence of females.

Developmental Competence of Oocytes Fertilized With Sperm Aged in an Incubator for 19.5 h

Frozen-thawed sperm were Percoll-purified and aged for 19.5 h at 38.5°C in 5.5% CO_2 and air in 500 μL modified IVF-TALP (750,000 sperm/mL; 0.6% BSA, 1.0 mM sodium pyruvate, 21.5 mM lactic acid, and 50 μg/mL penicillin-streptomycin). Sperm from 4 bulls of 2 different breeds were used, 2 bulls per replicate. Forty to sixty COCs, presumed mature, were then added to each well of aged sperm. Heparin (1.75 U/mL) was added to facilitate capacitation of sperm along with PHE. To provide a control, additional sperm were thawed and Percoll-purified at the conclusion of maturation of oocytes and added to remaining COCs in a separate 4-well plate, along with PHE. Putative zygotes were denuded of associated cumulus and spermatozoa 10-19 h post-fertilization and cultured in KSOM. Cleavage rate and development to blastocyst were assessed on days 3 and 8 post-fertilization, respectively. Day 9 blastocysts were stained with Hoechst 33342 (5 μg/mL) to assess total number of nuclei. Stained blastocysts were exposed to UV light and evaluated using a Nikon Eclipse TE300 inverted microscope fitted with a uv-2A filter (light exciter, 330 nm; dichroic, 400 nm; and barrier, 420 nm). Experiment was replicated 3 times with 377-385 oocytes included in each treatment.

Developmental Competence of Oocytes Fertilized With Sperm Aged 8 or 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C

Cumulus oocyte complexes were matured and then fertilized with sperm treated in the following manner: sperm Percoll-purified within 1 h post-thaw (control); sperm aged in artificial insemination (AI) straws for 8 or 14 h post-thaw at 34.4°C in a H_2O bath prior to Percoll-purification; or sperm aged in an AI straw for 23 h post-thaw at 4°C in a refrigerator prior to Percoll-purification. Proportion of motile sperm was assessed before and for some replicates, after Percoll-purification. Sperm from 9 bull of various breeds were used individually or in combination with sperm from a second bull. Putative zygotes were denuded of associated cumulus and spermatozoa and cultured for 9 days post-fertilization at which time blastocysts were stained with Hoechst 33342 to determine number of nuclei. Experiment was replicated 9 times with 180-546 oocytes included in each treatment.

Sex Ratio of Embryos Produced Using Sperm Aged 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C

Cumulus oocyte complexes were matured and then fertilized with sperm treated in the following manner: sperm Percoll-purified within 1 hour post-thaw (control); sperm aged in an AI straw for 14 h post-thaw at 34.4°C in a H_2O bath prior to Percoll-purification; or sperm aged in an AI straw for 23 h post-thaw at 4°C in a refrigerator prior to Percoll-purification. Proportion of motile sperm was assessed before Percoll-purification. Sperm from 4 different bulls, representing 3

different breeds (Senepol, Simmental, and Angus), were evaluated, 1 bull per replicate. Putative zygotes were denuded of associated cumulus and spermatozoa 17-18 h post-fertilization and cultured for 43-68 h. Cleaved embryos were sexed and blastomere number recorded. Cleavage rate and development of 8-16 cell embryos were recorded and not included in developmental study previously described. Experiment was replicated 4 times with 358-453 oocytes included in each treatment.

Sex Ratio of Embryos Produced Using Sperm Aged 8 or 14 h in a Water Bath at 34.4°C

Cumulus oocyte complexes were matured and then fertilized with sperm treated in the following manner: sperm Percoll-purified within 1 hour post-thaw (control); or sperm aged in an AI straw for 8 or 14 h post-thaw at 34.4°C in a H_2O bath prior to Percoll-purification. Proportion of motile sperm was assessed before Percoll-purification. Sperm from 3 different bulls, representing 2 different breeds (Holstein and Senepol), were evaluated, 1 bull per replicate. Putative zygotes were denuded of associated cumulus and spermatozoa 10-19 h post-fertilization and cultured for 67 h. Cleaved embryos were sexed and blastomere number recorded. Cleavage rate and development of 8-16 cell embryos were recorded and not included in developmental study previously described. Experiment was replicated 3 times with 195-233 oocytes included in each treatment.

Statistical Analysis

For all experiments, data were arranged in a randomized block design and analyzed using mixed models of SAS (2000). Least square means were presented in percentage format where applicable for ease of comparison to literature. Normality of data was tested using the Shapiro-Wilk test. When normality values were less than 0.85, data were log transformed.

CHAPTER IV

RESULTS

Developmental Competence of Oocytes Fertilized With Sperm Aged in an Incubator for 19.5 h

Aging sperm in a incubator did not influence proportion of putative zygotes recovered and placed in culture after denuding (table 1; P>0.1; SEM=12.4). Aging sperm did reduce ability of oocytes to cleave after fertilization when compared to those fertilized with control sperm (table 1; P<0.05; SEM=8.0). However, ability of cleaved embryos to develop to 8-16 cell (table 1; P>0.1; SEM=8.5) or blastocyst (table1; P>0.1; SEM=4.6) was not compromised.

Table 1: Developmental potential of oocytes fertilized with control sperm or sperm aged 19.5 h before fertilization

Sperm	Reps	No. Oocytes	PZ Recovered (%)	Cleaved (%)	8-16 Cell (%)	Blastocyst (%)
Control	3	377	219(58.7)	186(87.1)[a]	157(84.3)	43(23.2)
Aged	3	385	224(60.4)	140(65.4)[b]	86(63.5)	24(18.3)
P-Value	----	----	P=0.3456	P=0.0284	P=0.2269	P=0.4448
SEM	----	----	12.4	8.0	8.5	4.6

[ab]Least square means with different superscripts differ
Reps: total number of replicates
No. of oocytes: number of oocytes placed in maturation medium
PZ Recovered: number of putative zygotes placed in culture after denuding of oocytes expressed as proportion of oocytes placed in maturation medium
Cleaved: number & proportion of putative zygotes cleaved
8-16 cell: number & proportion of cleaved embryos developing to 8-16 cell
Blastocyst: number & proportion of cleaved embryos developing to blastocyst

Developmental Competence of Oocytes Fertilized With Sperm Aged 8 or 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C

Aging of sperm for 14 h in a water bath or 23 h in a refrigerator reduced proportion of motile sperm (table 2; P<0.0001; SEM=4.1). Proportion of motile sperm over all treatments increased after Percoll-purification (53.0 vs. 64.6%; P<0.03; SEM=5.1; data not shown). However, proportion motile was still lower for sperm aged 14 h in a water bath than control and refrigerated (table 2; P<0.07; SEM=8.8).

Table 2: Progressive motility of control sperm and sperm that were aged 8 or 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C

Aging of Sperm	Reps	Motility After Aging (%)	Motility After Purification (%)
Control	11	69.2^{a}	77.3^{a}
8 h Water bath	4	64.1^{ab}	----
14 h Water bath	8	42.1^{c}	48.9^{b}
23 h Refrigerator	5	56.8^{b}	69.3^{ab}
P-Value	----	P<0.0001	P=0.0660
SEM	----	4.1	8.8

^{abc}Least square means with different superscripts differ
Reps: total number of replicates
Motility After Aging: proportion of motile sperm assessed after aging of sperm in AI straw but before Percoll-purification
Motility After Purification: proportion of motile sperm assessed after Percoll-purification

Proportion of PZ recovered after denuding was similar indicating minimal effects of adding aged sperm to oocytes (table 3; P>0.5; SEM=6.8). Fertilization of oocytes with sperm aged 14 h in a water bath reduced ability of putative zygotes to cleave (table 3; P<0.05; SEM=6.4); however, ability of cleaved embryos to develop to 8-16 cell (table 3; P>0.1; SEM=3.8) or blastocyst (table 4; P>0.1; SEM=6.6) was not altered when oocytes were fertilized with aged sperm.

Table 3: Developmental potential of oocytes fertilized with control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C

Aging of Sperm	Reps	No. Oocytes	PZ Recovered (%)	Cleaved (%)	8-16 Cell (%)
Control	9	546	310(60.9)	240(76.2)[a]	212(89.9)
8 h Water bath	4	180	106(59.2)	73(72.6)[ab]	64(85.4)
14 h Water bath	7	403	240(56.7)	135(59.9)[b]	114(81.9)
23 h Refrigerator	4	213	233(67.8)	190(76.8)[a]	163(88.3)
P-Value	----	----	P=0.6824	P=0.0282	P=0.3130
SEM	----	----	6.8	6.4	3.8

[ab]Least square means with different superscripts differ
Reps: total number of replicates
No. Oocytes: number of oocytes placed in maturation medium
PZ Recovered: number of putative zygotes placed in culture after denuding of oocytes expressed as proportion of oocytes placed in maturation medium
Cleaved: number & proportion of putative zygotes cleaved
8-16 cell: number & proportion of cleaved embryos developing to 8-16 cell

Table 4: Development to blastocyst and number of nuclei for oocytes fertilized with control sperm or sperm that were aged 8 o r 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C[†]

					Average Number of Nuclei			
Aging of Sperm	Reps	Blast (%)	Total Stained	Reps	NB	XB	HgB	HB
Control	9	74(31.4)	14	4	31.2	83.7	153.6	----
8 h Water bath	4	26(32.4)	----	0	----	----	----	----
14 h Water bath	7	53(45.1)	15	4	----	101.0	126.0	161.1
23 h Refrigerator	4	50(30.2)	18	3	----	90.6	138.0	----
P-Value	----	P=0.1494	----	----	Statistics not performed due to			
SEM	----	6.6	----	----	few number of blasts stained			

[†]Blastocysts stained day 9 post IVF; No blastocysts were stained for sperm aged 8 h
[ab]Least square means with different superscripts differ
Reps: total number of replicates
Blast: Blastocysts; proportion of cleaved embryos developing to blastocyst
NB: normal blastocyst; XB: expanded blastocyst; HgB: hatching blastocyst; HB: hatched blastocyst

Total number of nuclei was determined in a subset of blastocysts originating from control and aged sperm; however, statistical analysis was not applied due to small sample sizes (table 4).

Sex Ratio of Embryos Produced Using Sperm Aged 14 h in a Water Bath at 34.4°C or 23 h in a Refrigerator at 4°C

Fertilization of oocytes with sperm aged 14 h in a water bath increased proportion of female embryos as compared to those fertilized with control sperm or sperm that had been refrigerated 23 h (figure 3; $P<0.05$; SEM=6.6). In this experiment, aging sperm for 14 h in a water bath reduced proportion motile when compared to control or sperm aged 23 h in a refrigerator (table 5; $P<0.005$; SEM=5.4). However, fertilizing oocytes with aged sperm did not affect proportion of putative zygotes recovered after denuding (table 5; $P>0.5$; SEM=4.9). Analysis of embryo development after fertilization revealed similar results to those reported previously in the development study. Aging sperm specifically for 14 hours in a water bath reduced ability of putative zygotes to cleave (table 5; $P<0.005$; SEM=5.8), however, ability of cleaved embryos to develop to 8-16 cell was not affected for either sperm aging treatment (table 5; $P>0.1$; SEM=11.8). Number of blastomeres comprising embryos originating from aged sperm was similar to controls (table 6; $P>0.5$; SEM=1.3). Moreover, blastomere number was similar regardless of the sex of the embryo (table 6; $P>0.5$; SEM=1.1).

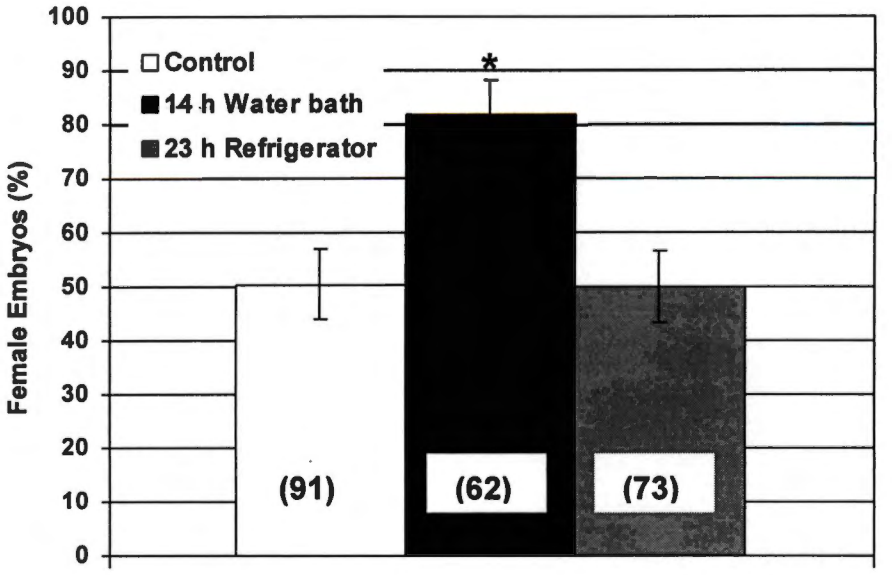


Figure 3: Sex ratio of cleavage stage embryos produced using control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C ($P<0.05$; SEM=6.6); total embryos sexed indicated in parentheses; asterick indicates statistical significance.

Table 5: Developmental competence of oocytes fertilized with control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C

Aging of Sperm	Reps	Motility (%)	No. Oocytes	PZ Recovered (%)	Cleaved (%)	8-16 Cell (%)
Control	4	67.5[a]	195	125(63.5)	91(72.0)[a]	60(63.8)
14 h Water bath	4	37.1[b]	180	162(59.1)	69(37.9)[b]	43(46.8)
23 h Refrigerator	3	61.9[a]	268	114(61.2)	79(62.4)[a]	54(61.1)
P-Value	----	P=0.0029	----	P=0.7365	P=0.0029	P=0.1661
SEM	----	5.4	----	4.9	5.8	11.8

[ab]Least square means with different superscripts differ
Reps: total number of replicates
Motility: proportion of motile sperm assessed before Percoll-purification
No. Oocytes: number of oocytes placed in maturation medium
PZ Recovered: number of putative zygotes placed in culture after denuding of oocytes expressed as proportion of oocytes placed in maturation medium
Cleaved: number & proportion of putative zygotes cleaved
8-16 Cell: number & proportion of cleaved embryos developing to 8-16 cell

Table 6: Blastomere number comprising embryos produced using control sperm or sperm that were aged 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C[†]

Aging of Sperm	Reps	Total Sexed	No. Male Embryos	No. Female Embryos	Average Blastomere Number		
					Males	Females	Overall
Control	4	91	46	45	7.4	5.9	6.7
14 h Water bath	4	62	14	48	6.6	5.9	6.2
23 h Refrigerator	3	73	39	34	7.1	6.9	7.0
P-Value	----	----	----	----	P=0.5924		P=0.5607
SEM	----	----	----	----	1.3		1.1

[†]Blastomere number assessed 43-67 hours post IVF at time of sexing
Reps: total number of replicates
Average Blastomere Number: average number of blastomeres per embryo according to sex and treatment

Sex Ratio of Embryos Produced Using Sperm Aged 8 or 14 h in a Water Bath at 34.4°C

Fertilization of oocytes with sperm aged 14 h in a water bath tended to increase proportion of female embryos when compared to control sperm, whereas sperm aged 8 h did not affect proportion of female embryos (figure 4; $P<0.1$; SEM=9.7). In this experiment, aging sperm for either 8 or 14 h in a water bath did not reduce proportion motile compared to control sperm (table 7; $P>0.1$; SEM=10.0). Fertilization of oocytes with aged sperm did not affect proportion of putative zygotes recovered after denuding (table 8; $P>0.1$; SEM=5.5). In contrast to previous experiments, fertilization of oocytes with aged sperm, either 8 or 14 h in a water bath, did not affect ability of putative zygotes to cleave (table 7; $P>0.5$; SEM=14.6) or develop to 8-16 cell (table 7; $P>0.1$; SEM=7.3) when compared to control. Number of blastomeres comprising embryos originating from aged sperm was similar to controls (table 8; $P>0.5$; SEM=1.5). Moreover, blastomere

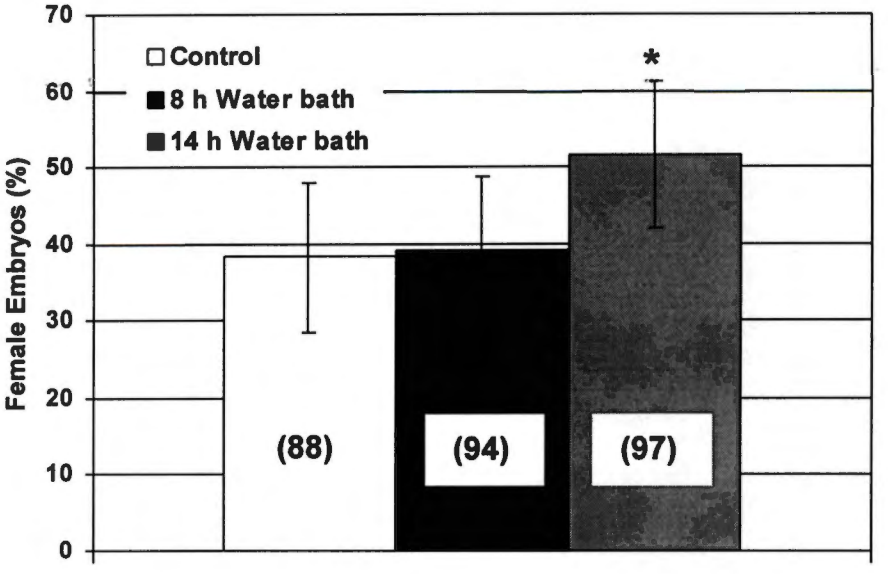


Figure 4: Sex ratio of cleavage stage embryos produced using control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C (P=0.08; SEM=9.7); total embryos sexed indicated in parentheses; asterick indicates statistical significance.

Table 7: Developmental competence of oocytes fertilized with control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C

Aging of Sperm	Reps	Motility (%)	No. Oocytes	PZ Recovered (%)	Cleaved (%)	8-16 Cell (%)
Control	3	72.7	195	131(68.7)	89(68.5)	73(85.2)
8 h Water bath	3	65.7	195	142(75.2)	101(70.2)	90(90.8)
14 h Water bath	3	45.0	233	140(58.8)	98(70.1)	78(84.8)
P-Value	----	P=0.2381	----	P=0.1965	P=0.9792	P=0.4628
SEM	----	10.0	----	5.5	14.6	7.3

Reps: total number of replicates
Motility: proportion of motile sperm assessed before Percoll-purification
No. Oocytes: number of oocytes placed in maturation medium
PZ Recovered: number of putative zygotes placed in culture after denuding of oocytes expressed as proportion of oocytes placed in maturation medium
Cleaved: number & proportion of putative zygotes cleaved
8-16 Cell: number & proportion of cleaved embryos developing to 8-16 cell

number was similar regardless of the sex of the embryo (table 8; P>0.1; SEM=1.5).

Table 8: Blastomere number comprising embryos produced using control sperm or sperm that were aged 8 or 14 h in a water bath at 34.4°C[†]

Aging of Sperm	Reps	Total Sexed	No. Male Embryos	No. Female Embryos	Average Blastomere Number		
					Males	Females	Overall
Control	3	88	54	34	9.7	9.1	9.4
8 h Water bath	3	94	57	37	9.2	8.8	9.0
14 h Water bath	3	97	45	52	8.5	8.1	8.3
P-Value	----	----	----	----	P=0.9737		P=0.2140
SEM	----	----	----	----	1.5		1.5

[†]Blastomere number assessed 66-68 hours post-IVF at time of sexing
Reps: total number of replicates
Total sexed: total number of cleaved embryos for which sex was determined
Average Blastomere Number: average number of blastomers per embryo according to sex and treatment

Effect of Aging on Motility of Sperm

Effect of aging on motility of sperm before and after Percoll-purification was analyzed for all treatments over the entire study. Aging sperm for 14 h in a water bath or 23 h in a refrigerator reduced proportion motile when compared to control or sperm aged 8 h (table 9; P<0.0001; SEM=3.3). After purification, proportion of motile sperm tended to remain lower for sperm aged 14 h (table 9; P=0.07; SEM=8.8). Motility was not assessed after Percoll-purification for sperm aged 8 h.

Effect of Sex on Blastomere Number

Number of cells per embryo was analyzed for effect of sex over the entire study (table 10). Number of blastomeres comprising male or female embryos did not differ (P>0.1; SEM=1.5).

Table 9: Effect of aging on proportion of motile sperm

Aging of Sperm	Reps	Motility Before Percoll (%)	Reps	Motility After Percoll (%)
Control	18	69.4[a]	6	77.3[a]
8 h Water bath	7	64.7[ab]	0	----
14 h Water bath	16	41.4[c]	4	48.9[b]
23 h Refrigerator	9	58.2[b]	6	69.3[ab]
P-Value	----	P<0.0001	----	P=0.0660
SEM	----	3.3	----	8.8

[abc]Least square means with different superscripts differ
Reps: number of replicates for which motility before or after Percoll-purification was assessed
Motility Before Percoll: proportion of motile sperm assessed after aging of sperm in AI straws but before Percoll-purification
Motility After Percoll: proportion of motile sperm assessed after Percoll-purification

Table 10: Effect of sex on blastomere number

Sex	Reps	# Embryos	Number of Blastomeres
Male	7	197	8.0
Female	7	214	7.4
P-Value	----	----	P=0.2099
SEM	----	----	1.0

Number of Blastomeres: determined at time of sexing
Reps: total number of replicates
Embryos: total number of male and female embryos for which blastomere number was determined

CHAPTER V

DISCUSSION

Aging sperm for 14 h in a water bath or 23 h in a refrigerator reduced proportion of motile sperm and compromised ability of oocytes to cleave following fertilization. However, of those embryos that cleaved, development to 8-16 cell and blastocyst was not compromised. Surprisingly, fertilization of oocytes with sperm aged for 14 h in a water bath post-thaw altered sex ratio of resulting embryos by increasing the proportion of females.

Production of Developmentally Competent Embryos From Sperm Aged Post-Thaw

Effects of aging sperm post-thaw on motility of sperm

Aging sperm prior to fertilization, whether in a water bath for 14 h or in a refrigerator for 23 h, reduced proportion of motile sperm. Negative effect of aging sperm for reducing motility was not surprising, as this effect has previously been characterized (reviewed by Foote and Parks, 1993; Vishwanath and Shannon, 1997). In fact, it is recommended that frozen sperm be inseminated within 15 minutes post-thaw to minimize loss of motility and fertility (American Breeders Service A.I. Management Manual, 1991).

Proportion of motile sperm was reduced to a much greater extent for sperm aged 14 h in a water bath than sperm aged 23 h in a refrigerator. This was most likely due to effects of environment in which aging was performed, specifically

differences in temperature. Metabolism of sperm occurs at greater rates in higher temperatures (Blackshaw et al., 1957), leading to the build-up of waste products such as reactive oxygen species, which in turn could damage membranes (Vishwanath and Shannon, 1997). Kelly and coworkers (1962) stored frozen-thawed bovine semen in ice water (1°C) for 4 or 6 hours with little to no affect on non-return rates. Thus, sperm aged in a refrigerator may incur less damage and sustain motility longer than sperm aged in a water bath at 34.4°C.

Proportion of motile sperm was not reduced for sperm aged 8 h post-thaw in a water bath versus sperm aged 14 h. This was surprising as effect of aging on sperm motility was thought to be time dependent. Due to the restricted endogenous synthetic ability of sperm (Fraser, 1992), metabolism cannot be sustained indefinitely. Therefore, motility cannot be sustained indefinitely. Aging for longer periods of time, 14 h versus 8 h, may cause cessation of metabolic processes, and thus cessation of motility in many spermatozoa.

Purification through Percoll increased proportion of motile sperm; however, motility still remained lower for sperm aged 14 h in a water bath. Percoll-purification is intended to remove sperm that may be damaged morphologically and non-motile (Pousette et al., 1986). However, for semen samples in which large numbers of sperm are damaged, purification may be compromised.

Incubation of aged sperm with oocytes, as was done for fertilization in this thesis, may compromise the quality of oocytes. For example, leakage of factors

such as digestive enzymes could possibly compromise oocyte membranes. Visual damage to oocytes would increase cull rate of putative zygotes at denuding. However, no effect of aged sperm on quality of oocytes was observed in this thesis.

Effects of aging sperm post-thaw on embryonic development after fertilization

Fertilizing ooctyes with aged sperm, whether in an incubator for 19.5 h or in a water bath for 14 h, reduced proportion of putative zygotes that cleaved. This was expected, as reduced fertility of aged sperm has previously been reported (Tesh, 1969; Martin-DeLeon and Boice, 1982; reviewed by Vishwanath and Shannon, 1997).

Reduced cleavage could be attributed to reduced fertilization by aged sperm or a reduced ability of aged sperm to complete fertilization and produce a functional zygote. Effects of aging (reviewed by Vishwanath and Shannon, 1997) include oxidative damage to sperm membranes. If damaged sperm remain motile, binding to oocytes may be difficult. A reduction in proportion of motile sperm, as observed after aging, would reduce the number of sperm capable of fertilization on their own. In this thesis, sperm were added to oocytes in equal numbers between treatments without adjusting for reduced motility. Thus, oocytes fertilized with aged sperm actually received fewer motile sperm. Sperm-egg ratios in this thesis were approximately 7,500:1. Sperm-egg ratios below

5,000:1 reduced fertilization rate below 50% as determined by presence of two pronuclei (Kroetsch and Stubbings, 1992).

Reduced cleavage rate could stem from an increased proportion of non-functional zygotes. Breakdown of the fertilization process may have occurred after initiation of fertilization by the spermatozoon but short of cellular division. Zygotes produced using sperm aged in vitro (0-6, 9-15 and 18-24 hours) have been reported to exhibit a reduced ability to reach prometaphase (Smith and Lodge, 1987). Nuclear membranes could be damaged by oxygen radicals, allowing them access and potential damage to DNA (reviewed by Vishwanath and Shannon, 1997). Damage to DNA may prevent successful incorporation and/or function of the male genome.

It was surprising that developmental potential of cleaved embryos after fertilization of oocytes with aged sperm was comparable to that of control embryos. However, development was only observed to blastocyst. It remains to be determined if these embryos could establish a pregnancy and produce healthy offspring. Perhaps a more thorough investigation of these embryos, such as chromosome preparations, may reveal developmental problems similar to observations made by Martin-DeLeon and Boice (1982).

It must be pointed out that the duration during which sperm were aged was within the 24-48 h period that bovine sperm remain fertile within the oviduct (reviewed by Austin, 1975). It is unclear whether frozen-thawed sperm have a shortened fertile lifespan. The extender in which sperm are held may influence

the time sperm remain viable post-thaw. For example, Vishwanath and Shannon (1997) reported that frozen-thawed semen that had been re-diluted in a typical diluent was only fertile for a few hours, whereas semen re-diluted in CAPROGEN® remained fertile for inseminations performed during an entire working day (reviewed by Vishwanath and Shannon, 2000). Harmful effects of aging reported by others (Tesh, 1969; Martin-DeLeon and Boice, 1982) may have been due to excessive aging outside the normal fertile lifespan of sperm.

It was also surprising to observe embryonic development comparable to that of controls after fertilization of oocytes with either sperm aged 14 h in a water bath or sperm aged 19.5 h in an incubator. The environments in which each was aged differed greatly. It was presumed sperm aged within an AI straw would survive to a greater extent and yield embryos of greater developmental potential than sperm aged in a culture dish in an incubator. Sperm aged in an incubator were exposed to high levels of oxygen (20%). Oxygen radicals in the environment can have negative effects on motility and membranes of sperm, including mitochondrial membranes (reviewed by Vishwanath and Shannon, 1997). Without a method of mitochondria turnover, sperm may suffer greatly from degeneration of mitochondria (reviewed by Vishwanath and Shannon, 1997).

In contrast, sperm aged within AI straws, as described in this thesis, were within a closed environment surrounded by components of semen extender and seminal fluid. One may assume the microenvironment within an AI straw is

anaerobic. Without an influx of fresh oxygen to serve as an electron acceptor, mitochondrial respiration may slow down without the continual downstream pull of electrons by oxygen (reviewed by Gnaiger et al., 1995). Therefore, metabolism of sperm in AI straws may have slowed and diverted to more of an anaerobic mode due to lack of available oxygen. A lowered metabolism rate, through perhaps fermentation, would decrease the amount of reactive oxygen species produced, thus decreasing the amount of damage sustained by such molecules (reviewed by Vishwanath and Shannon, 1997). The inclusion of cryoprotectants within semen extender may also render protection for sperm membranes during such a period, as they are intended to protect sperm from the freezing process (reviewed by Curry, 2000).

Fertilization of Oocytes With Sperm Aged Post-Thaw May Alter Sex Ratio

Aging sperm for 14 h post-thaw in a water bath at 34.4°C increased the proportion of female embryos after fertilization of oocytes. Several researchers have reported altered sex ratios after manipulating time of insemination (Verme and Ozoga, 1981; Wehner et al., 1997; Pursley et al., 1998). Specifically, more females were produced when inseminations were performed early, prior to ovulation. One could speculate that this would cause aging of the sperm, which in turn altered sex ratio of offspring. Results of this thesis may be in agreement with that hypothesis.

Alterations in sex ratio, as observed with aging sperm 14 hours in a water bath at 34.4°C, may be explained by one of the following hypotheses. It is

possible that biological differences between X- and Y-bearing sperm, other than DNA content, are revealed through aging, resulting in greater fertilization by X-bearing sperm. Such differences may be attributed to haploid gene expression (reviewed by Erickson et al. 1981; Lele and Wolgemuth, 1998). However, the X and Y chromosomes are believed to be heterochromatic and thus transcriptionally inactive by the pachytene substage of meiosis, as they are separated in a sex vesicle, or body, during prophase of meiosis (McKee and Handel, 1993).

Differences in gene expression between X- and Y-bearing sperm should be eliminated due to the presence of intercellular bridges between developing spermatids. Sharing of protein and mRNA between spermatids has been demonstrated (reviewed by Hendriksen, 1999). Transcription after disruption of intercellular bridges may be ruled out, because in the mouse, gene transcription stops when DNA packing becomes too tight to allow synthesis of mRNA (reviewed by Hendriksen, 1999).

However, evidence suggests reactivation of several Y-linked genes, as well as some X-linked genes, post-meiotically (Hendriksen et al., 1995; reviewed by Hendriksen, 1999). Post-meiotic expression may result in greater fertilization rate for X-bearing sperm over Y-bearing. Bredbacka and Bredbacka (1996) suggested X-linked genes hypoxanthine phosphoribosyl transferase and glucose-6-phosphate dehydrogenase, which are involved with regulating levels of

reactive oxygen species, may confer an increased ability to withstand oxidative stress to X-bearing sperm.

Differential gene expression in X- and Y-bearing spermatids could translate into biological differences if sharing of such gene products was prevented in some manner. Many proteins are transported vectorially directly to their pre-determined location in a cellular organelle or structure (reviewed by Bellve, 1982). Proteins can be cotranslationally discharged into the lumen of the endoplasmic reticulum (ER) or inserted into the membrane and then translocated by directional mechanisms to ensure they reach their intended destination (reviewed by Bellve, 1982). The ER is highly developed in spermatocytes and spermatids and becomes increasingly complex during early spermiogenesis, even extending into the intercellular bridges (reviewed by Bellve, 1982). Given such complexity, it's been suggested that functional integration of the ER and the nuclear matrix could facilitate vectorial translocation of sex chromosome-linked post-meiotic gene products in sperm (reviewed by Bellve, 1982).

Support for non-sharing of gene products is provided by several examples (reviewed by Hendriksen, 1999). The *t*-allele in mice has been shown to exhibit a distorted transmission ratio where heterozygous males transmit the t complex to more than 90% of their offspring, (reviewed by Hendriksen, 1999). Increased fertilization rate by t complex-containing sperm suggests unequal sharing of this gene product (reviewed by Hendriksen, 1999). A second example was described in mice that exhibit a chromosome 6 to 16 translocation that results in fertilization

by twice the number of Y-bearing sperm than X-bearing (reviewed by Hendriksen, 1999). A third example involves mice with a partial deletion of the long arm of the Y chromosome, causing a higher frequency of abnormal sperm heads, that results in a sex ratio distortion in favor of females (reviewed by Hendriksen, 1999).

Another potential explanation for the production of more female embryos from aged sperm involves potential alterations in pH. In the anaerobic environment within an AI straw, sperm metabolism converts to utilize fructose, which yields lactic acid when metabolized (Graves et al., 1966). A build up of lactic acid may reduce the pH within the straw. According to Shettles (1970), a drop in pH caused by a build up of lactic acid may facilitate selection of X-bearing sperm for the production of females, as seen in the present thesis. However, this concept has been disputed by studies in which no effect of pH on sex ratio or survivability of sperm was observed (Downing and Black, 1976; Muehleis and Long, 1976).

One final consideration in explaining altered sex ratio in favor of females after fertilization with aged sperm would be an increased amount of oocyte activation, termed parthenogenesis. If aging compromised sperm membranes, the possibility exists that factors released from damaged sperm could in some way initiate parthenogenesis, inflating the proportion of female embryos.

Effect of parthenogenesis for skewing sex ratio would be greatest in situations of low cleavage rate. In the present thesis, variability among bulls and the ability of their sperm to withstand aging often yielded low cleavage rates. In fact,

replicates in which the greatest shift in sex ratio was observed, the greatest reduction in cleavage rate was exhibited. The following would be an example. Assuming a 10% parthenogenic rate for 100 PZ, 10 of the resulting females would be parthenotes. If cleavage rate was 30% (30 cleaved) and the sex ratio was 90% female (27/30), only 17 of those embryos sexed as females were truly females as determined by fertilization by sperm, resulting in an actual sex ratio of 57% (17/30).

In the second sex ratio experiment, sex ratios for control as well as sperm aged 8 and 14 h were all below the expected 50:50. This was due to extreme variation between bulls. In particular, sperm from one bull yielded a control sex ratio of 20% female. This effect obviously carried over to the other treatments. However, there still remained a slight trend of more females from sperm aged 14 h.

Sex ratio of embryos following fertilization was not only affected by aging, but the manner in which sperm were aged. Sperm aged in a refrigerator 23 h did not alter sex ratio of embryos from that of control. Effect of aging sperm for 23 h in a refrigerator on motility was minimal compared to control. In this scenario, X- and Y-bearing sperm may have been able to survive equally as well, resulting in a sex ratio not different from control. Additionally, aging sperm for 23 h in a refrigerator did not reduce cleavage rate. This further supports a theory of equal survival and thus fertilization for X- and Y-bearing sperm.

Aging sperm only 8 h in a water bath also did not alter sex ratio of embryos after fertilization. This was surprising as it was presumed aging would alter sex ratio in a time-dependent manner. Results may suggest that in order for aging to reveal differences between X- and Y-bearing sperm, duration of aging must be more extreme than 8 h. Aging sperm 8 h in a water bath did not reduce proportion of motile sperm nor the cleavage rate of PZ after fertilization. These results further suggest that aging 8 h in a water bath did not cause differential survival and/or fertilization by X- or Y-bearing sperm, which would have resulted in an altered sex ratio.

No statistical difference in cell number was observed between male and female embryos day 3 post-fertilization. These results were in contrast to several studies reporting a difference in cell number between male and female bovine embryos (Yadav et al., 1993; Bredbacka and Bredbacka, 1996). The above studies observed differences within the first two days following fertilization. The period of development at which the bovine embryo reaches the 8-16 cell stage is a prolonged phase in which DNA synthesis and transition from maternal to zygotic control of development occurs (First and Parrish, 1987). In the bovine, an 8-cell embryo is observed d 3 post-fertilization (reviewed by McLaren, 1974), which was when sexing was performed in this thesis. Thus, it remains possible this period allowed female embryos to catch up to male embryos, with regard to cell number.

One potentially limiting factor of sex ratio studies performed within this thesis was the method in which sex of embryos was determined. A PCR kit that amplified non-disclosed regions specific to the Y chromosome was used which allowed direct visualization of amplified male DNA within reaction tubes. Female embryos should not have fluoresced. Advantages of this kit include amount of time and work saved in not having to run DNA samples on gels. However, lack of an internal control such as co-amplification of an X chromosome-specific or autosomal gene, prevented discrimination of tubes containing female embryos from those containing male embryos that failed to undergo amplification. Visualization of blastomeres within each tube was performed to guarantee transfer of sample to PCR tubes. Despite such concerns, the manufacturer of this kit claims an accuracy rate for sex determination of 99%.

Summary and Implications

Aging sperm 14 h in a water bath at 34.4°C or 23 h in a refrigerator at 4°C reduced proportion motile and compromised ability of oocytes to cleave following fertilization. However, of the embryos that cleaved, subsequent development of to 8-16 cell or blastocyst was not compromised. Sex ratio of embryos produced using sperm aged 14 h in a water bath was altered, yielding more female embryos. Results suggest the possibility that biological differences may exist between X- and Y-bearing sperm. If results are valid, such differences between X- and Y-bearing sperm may be exploited for the development of alternative methods of sex selection.

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