

Influencing Sex Ratio of Offspring in Dairy Cattle

Melissa Macfarlane, Angela Maschari, and Richard Pursley

This presentation focuses on data collected during the past 3 years that indicates the possibility that the amount of time that sperm live in the reproductive tract of a cow prior to ovulation can result in the deviation of the ~1:1 expected sex ratio of offspring. Data from these experiments cannot be included in these proceedings since they are not yet published. Therefore, in these proceedings, we would like to share with you our hypothesis as to why we think this phenomenon is occurring: “X sperm have a longer functional survival such that they have an advantage over Y sperm in binding to oviductal epithelial cells, remaining bound to oviductal epithelial cells over time, or both” and a review of literature that discusses what we know about the differences in X and Y chromosome bearing sperm that, in part, led us to this hypothesis.

Introduction

Bovine spermatozoa are the male gametes and are responsible for contributing half the offspring’s genetic information. Each spermatozoon contains 29 autosomes and one sex chromosome. X-chromosome-bearing sperm (X sperm) produce females and Y-chromosome-bearing sperm (Y sperm) produce males. Sperm are introduced into the female reproductive tract, either in the vagina during natural mating or in the uterus during artificial insemination. Upon introduction sperm must travel from the lower reproductive tract to the oviducts where they form a reservoir in the isthmus and await ovulation. Sperm are released from the oviductal reservoir in response to oviductal secretions around the time of ovulation. Once ovulation has occurred one sperm must penetrate the oocyte for a successful fertilization.

X- and y-chromosome bearing sperm

Spermatozoa carry either an X chromosome (X sperm) and produce female offspring or a Y chromosome (Y sperm) and produce male offspring. Interest in manipulating the sex of offspring has led to a search for differences between X and Y sperm, methods to detect these differences, and methods to separate sperm based on these differences.

Differences between X- and Y-chromosome bearing sperm

Differences between X and Y sperm fall into two categories: different chromosomes and different genetic expression. Chromosomal differences refer to differences in DNA content and the kinds and numbers of genes. These differences may be manifested in different genetic expression which could cause differences in sperm structure, function, or both.

One possible point of variation between X and Y sperm is their cell surface characteristics. Early studies claiming differences in charge between X and Y sperm (Blottner, et al., 1994, Engelmann, et al., 1988, Ishijima, et al., 1991, Kaneko, et al., 1983a, Kaneko, et al., 1984a), have since been invalidated because they used a method to distinguish X and Y sperm that was later shown to be inaccurate (Vankooij and Vanoost, 1992). In 1991, Cartwright et al. found little differences in sperm using charge-sensitive

aqueous two-phase partition, a precise system that can be used to partition cells based on different surface molecules, though differences between X and Y sperm were not studied directly. When electrophoresis was used to separate sperm on the basis of charge no differences were found in the ratio of X and Y sperm (Checa, et al., 2002) or the sex of calves born (Hafs and Boyd, 1974).

Although no differences were found based on charge, Cartwright et al. (1991) did find two populations of sperm having different surface properties using charge-insensitive aqueous two-phase partition. These two populations showed heterogeneity of non-charged surface molecules. While it was thought that these two populations represented X and Y sperm, later studies using the same system found that the separation was not based on specific surface molecules on Y sperm that are not on X sperm or vice versa (Cartwright, et al., 1993). Many techniques have been used to search for sex-specific surface antigens and differences in surface proteins between X and Y sperm. Attempts to identify differences in surface proteins and antigens in populations of sperm sorted to contain either X or Y sperm by flow cytometry have failed thus far (Hendriksen, et al., 1996, Howes, et al., 1997). One drawback of these studies is the use of flow-sorted sperm. Cell surface proteins could have been removed from the sperm heads during the sorting process, thus differences in these proteins would not have been detected. Also, the techniques used may not have been able to detect differences in very small peptides such as the H-Y antigen (Hendriksen, 1999).

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

The nature of spermatogenesis makes it unlikely that differences in the cell surface of X and Y sperm exist. During the mitotic and meiotic divisions of spermatogenesis cytokinesis is not complete; the cells remain connected by intercellular bridges. These intercellular bridges allow sharing of gene products between X and Y sperm (Hendriksen, 1999). Gene transcription stops before the intracellular bridges are disrupted (Dym and Fawcett, 1971, Hendriksen, 1999, Morales and Hecht, 1994), making it unlikely that the X and Y sperm differences would occur as a result of gene transcription after the disruption of intracellular bridges. However, unequal gene product sharing could be a possible mechanism to create differences in the cell surfaces of X and Y sperm, though there is no direct evidence for this (Hendriksen, 1999).

The fact that the X chromosome is larger than the Y chromosome may require X sperm to have larger heads than Y sperm, to accommodate the extra chromatin. Evidence exists both for and against size differences between X and Y sperm (Chandler, et al.,

1999, Cui, 1997, Hossain, et al., 2001, Revay, et al., 2004, van Munster, et al., 1999). The experimental procedures to assess size differed in these studies, making them hard to compare. Another confounding factor is the use of different processing and staining techniques. Processing and staining procedures have been found to influence the size measurements of sperm heads (Foote, 2003), which may mask or create artificial differences between the two populations. The most recent studies using fluorescence in situ hybridization (FISH) on individual sperm have not found significant differences (Hossain, et al., 2001, Revay, et al., 2004). Differences in size between X and Y sperm are not generally accepted today

The differences in the amount of DNA in X and Y sperm could cause differences in their densities. While early studies on density seemed to show differences between X and Y sperm (Kaneko, et al., 1984b, Kaneko, et al., 1987, Kaneko, et al., 1983b) the use of an inaccurate method for detection of X and Y sperm has since invalidated these studies. Separation of sperm using density gradients have had mixed results (Andersen and Byskov, 1997, Kobayashi, et al., 2004, Lin, et al., 1998, Luderer, et al., 1982, Upreti, et al., 1988). Results of studies using Percoll density gradients are further confounded by speculation that the separation of sperm by Percoll may be related more to differences in motility between X and Y sperm rather than differences in density (Kobayashi, et al., 2004).

Besides morphological differences, X and Y sperm may also have functional differences. Sperm motility has long been suspected of varying between X and Y sperm. Methods to select highly motile sperm include Percoll density gradient centrifugation, swim-up, and albumin gradients. The ability of these methods to separate X and Y sperm based on motility has been studied extensively, with mixed results (Aribarg, et al., 1996, Beernink, et al., 1993, Chen, et al., 1997, De Jonge, et al., 1997, Han, et al., 1993a, Kobayashi, et al., 2004, Lin, et al., 1998, Madrid-Bury, et al., 2003, Pyrzak, 1994, Upreti, et al., 1988, Wang, et al., 1994). In stationary fluids, X and Y sperm swim at the same speed (Penfold, et al., 1998, Sarkar, et al., 1984) but differ in their linearity and straightness of path (Penfold, et al., 1998). However, *in vivo* sperm are not in stationary fluid, instead, they are in a complex flow environment (Sarkar, et al., 1984). When placed in a flow-stream, sperm shift to a straighter path of movement with a decreased angular velocity; thus a shift is four times more pronounced in X sperm (Sarkar, et al., 1984).

Aside from motility, sperm could differ in rates of survival or capacitation. Human sperm exhibit a differential functional survival between X and Y sperm, with Y sperm surviving longer under *in vitro* conditions (Van Dyk, et al., 2001). In cattle, the opposite seems to be true. When sperm were pre-incubated prior to *in vitro* fertilization more female hatched blastocysts were produced compared with fresh sperm (Lechniak, et al., 2003).

Sperm in the female reproductive tract

In order to fertilize an oocyte, sperm must travel from the site of insemination to the site of fertilization in the female reproductive tract. In cattle, sperm are deposited in the vagina during natural mating and in the uterus during artificial insemination.

Fertilization occurs in the ampullary-isthmic junction of the oviduct. Sperm must travel through the cervix (in naturally mated animals), into the uterus, through the utero-tubal junction and into the oviduct. Only a small percentage of the sperm that are inseminated make it to the site of fertilization (Scott, 2000, Suarez, 1998, Suarez, 2002). The process of sperm transport in the female reproductive tract is not just a simple migration from the site of insemination to the site of fertilization; it is a complex and dynamic process that includes phases of sperm distribution, the formation of sperm reservoirs, the modulation of sperm physiology and acquisition of fertilization competence, the ascent of competent sperm to the site of fertilization and the elimination of the non-fertilizing sperm population (Scott, 2000).

Sperm transport through the female reproductive tract

Sperm transport through the female reproductive tract begins with insemination. When natural mating occurs millions to billions of sperm are placed in the vagina of the female and must travel through the cervix, however when artificial insemination (AI) is used, the cervix is bypassed and sperm are placed directly into the uterus. For both natural mating and AI, most sperm are lost through retrograde movement out the vagina (Scott, 2000). From the site of deposition, sperm must travel to the oviduct to fertilize an oocyte.

Sperm transport occurs in two phases, the rapid transport phase and the prolonged transport phase (Hawk, 1983, Scott, 2000). In the rapid transport phase, sperm travel to the oviducts within minutes, however these sperm are usually moribund, dead, or disrupted and do not contribute to the fertilizing population (Scott, 2000). In the prolonged transport phase sperm migrate to the oviduct over a period of six to twelve hours (Dobrowolski and Hafez, 1970, Hunter and Wilmut, 1984, Scott, 2000, Wilmut and Hunter, 1984). Once in the lower isthmus of the oviduct, fertilization competent sperm do not progress further until the periovulatory period (Hunter and Wilmut, 1984, Scott, 2000).

The primary mechanisms of sperm transport are smooth muscle contractions of the female reproductive tract, ciliary beats, fluid currents, and sperm movement by flagellar activity (Hawk, 1983). Smooth muscle contractions are increased and stronger during estrus compared with other stages of the estrus cycle (Hawk, 1983). These contractions are primarily responsible for sperm movement through the uterus (Hawk, 1983, Katila, 2001). Spatial constraints, epithelial surface characteristics, and fluid secretions affect sperm motility. Only motile sperm can cross the cervix and the cervical mucus; motility may also be necessary for crossing the utero-tubal junction (Scott, 2000). Functional interactions between sperm and luminal fluids and epithelial surfaces of the female reproductive tract promote the selection of physiologically normal sperm (Scott, 2000).

During transport, sperm accumulate and are retained in some regions of the reproductive tract for prolonged periods of time. These regions are referred to as sperm reservoirs (Scott, 2000). While the cervix has been considered a sperm reservoir for many years because many sperm are found there during the prolonged phase of sperm transport,

the functional sperm reservoir is in the oviductal isthmus (Hunter and Wilmut, 1982). This is considered the functional reservoir because it is the one that is drawn on at the time of ovulation. Sperm that will interact with the oocyte proceed from this reservoir (Hunter, 2003, Hunter and Wilmut, 1982).

Effect of time on sex ratio

Possible mechanisms that may be responsible for the effect of time of insemination on sex ratio include differential transport of sperm through the female tract, differences in capacitation times or functional survival between X and Y sperm, preferential selection of sperm, or sex related embryo death. In humans more Y sperm remained functional after extended *in vitro* incubation at 37°C (Van Dyk, et al., 2001). It has been shown that capacitation method can have an effect on the prevalence of X and Y sperm (Barczyk, 2001). Longer capacitation times favor human Y sperm while shorter times favor X sperm (Barczyk, 2001). These findings indicate that in humans Y sperm have longer functional survival possibly due to different rates of capacitation between X and Y sperm. Extended *in vitro* incubation of bull sperm produced more female hatched blastocysts (Lechniak, et al., 2003), indicating that in cattle the X sperm has longer functional survival or delayed capacitation compared to the Y sperm.

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

In our lab, we plan to continue to test the idea that prolonged *in vivo* incubation of sperm increases the chances for a heifer calf. Other factors such as body condition and pregnancy losses may play a critical role in the determination of sex ratio at birth.