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An Overview of Sex Selection at Conception in Mammals

Review Article

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ABSTRACT

Although the mechanisms under which sex ratio is controlled in nature remain unknown, researchers have attempted to treat sperm in an effort to predetermine the sex of the offspring. The methods of sperm separation that have been repeatedly reported to isolate fractions rich in X- or Y-chromosome bearing sperm are serum albumin gradient swim-up and flow cytometry. However, the ability of the serum albumin gradient method to isolate Y-chromosome rich fractions and alter the sex ratio of offspring is still a subject of controversy. The flow cytometry technology used to separate X- and Y-bearing sperm into live fractions has been improved and publications showed that the procedure for the capacitation of flow cytometry sorted sperm might be successfully applied for fresh sperm in *in vitro* fertilisation (IVP) programs. In the case of sexing embryos the use of the polymerase chain reaction (PCR) is a service offered by several embryo transfer practitioners, but it is labor intensive and costly. In addition, biopsied embryos do not survive freezing very well. In the near future, PCR assays for use in the predetermination of the sex of offspring may become available. In combination with *in vitro* maturation (IVM), *in vitro* fertilisation (IVP) and embryo transfer techniques, it is very likely that sexed spermatozoa or embryo will be used widely and efficiently in mammals where higher numbers of spermatozoa or embryo are usually required. To be commercially viable, a method of embryo sexing must be highly efficient, simple and cheap. Although many livestock breeders request embryo sexing, it has not found widespread use especially in developing countries.

KEY WORDS embryo, livestock, selection, sex ratio, sperm.

INTRODUCTION

With synchronization, artificial intelligence (AI) and *in vitro* production (IVP) of embryos, a deviation in the expected sex ratio has been reported; the percentage of male embryos is > 50% (King *et al.* 1991; Avery *et al.* 1992; Marquant-Le-Guienne *et al.* 1992; Carvalho *et al.* 1996; Pegoraro *et al.* 1998; Kamga *et al.* 2005). Predetermination of the sex of offspring could have a significant impact on livestock breeding and production, particularly in selection programmes where the product (e.g. milk) comes from only one sex (De Vries *et al.* 2008).

Prediction of sex offer also an advantage in the situation where a large number of embryos are needed to establish a herd or flock of specified genotype compare to the introduction of an exotic breed or species. Because there has not been a method for predetermining sex of offspring, animal production and breeding programs for the exploitation of skewed sex ratios have not been developed (Shelton, 1990). There are two approaches to predetermine sex of offspring. One is to ensure fertilization of oocytes by X-bearing or Y-bearing sperm, and a variety of methods have been proposed to sorted spermatozoa according to X or Y chromosome content (Amann, 1989; De Graaf *et al.* 2009).

The second approach for the predetermination of sex of offspring is to select embryos of the required sex before transfer to recipients. Several protocols have been established for sexing embryos in farm animals, such as karyotyping (King, 1984), H-Y antigen detection (Anderson, 1987), X-linked enzymatic determination (Monk and Handyside, 1988) and based on the identification of the Y chromosome, such as sex-determining region Y (SRY), zinc finger protein, Y-linked (ZFY) and testis-specific protein Y-encoded (TSPY) genes, include *in situ* hybridization, Southern dot blotting, polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) (Miller, 1991; Bredbacka and Peippo, 1992; Gutiérrez-Adán *et al.* 1996; Ng *et al.* 1996; Sohn *et al.* 2002; Huang *et al.* 2007). Moreover, sex can be selected during insemination, *in vitro* maturation, *in vitro* fertilization and embryo transfer. Therefore, a review of these methods is presented.

Sperm sexing methods

Serum albumin layering

Ericsson *et al.* (1973) first reported that when human sperm were layered on columns of liquid albumin, ~ about 85% of the resulting sperm migrating to the lowest portions of the columns bore a Y chromosome.

The results of this study soon became controversial when Ross *et al.* (1975) and Evans *et al.* (1975) reported that serum albumin layering did not isolate fractions rich in Y-bearing sperm.

However, later IVF studies confirmed Ericsson *et al.* (1973) findings (Dmowski *et al.* 1979; Burstein and Schenker, 1985; Beernink and Ericsson, 1982; Perrone and Testart, 1985; Quinlivan *et al.* 1982; Beernink *et al.* 1993; Bernstein, 1998).

David *et al.* (1977), Ueda and Yanagimachi (1987), Han *et al.* (1993) and Reubinoff and Schenker (1996) have also reported that albumin layering alters the sex ratio of offspring ranging from 73 to 90% male. Ericsson (1989), Ericsson (1994) contended that technician error in procedure implementation is responsible for the contradictory results reported from other laboratories. Altered sex ratios have been reported in sheep using this technique (White *et al.* 1984).

However, in swine (Dixon *et al.* 1980) and horses (Goodeaux and Krieder, 1978), treatment of sperm with the Ericsson's method failed to produce altered sex ratios. In cattle, Ericsson *et al.* (1980) and Foote (1985) reported an altered sex ratio with treated sperm while Ferguson *et al.* (1976) and Beal *et al.* (1984) detected an unaltered sex ratio following serum albumin separated sperm. Amann (1989) postulated that serum albumin layering may not alter the proportion of Y-bearing sperm, but may alter the ability of Y-bearing sperm to fertilize the ovum.

Flow cytometric sorting

Flow sorting systems commercialization started in the 1970 s and developed rapidly in conjunction with the computer revolution since 1980 s. Although the primary application has been in medical research and diagnosis with respect to blood cells, flow cytometry is an effective tool for many types of cell suspensions (Sharpe and Evans, 2009). Flow cytometry is enabling scientists to measure the relative DNA content of individual sperm at a relatively rapid rate (Johnson, 1992; Checa *et al.* 2002). Fertility results following IVF with sex-sorted sperm yielded positive results with several farm species (Carvalho *et al.* 2010). Rath *et al.* (1993) reported that the sex ratio of piglets born following transfer of IVF-derived embryos was significantly altered. This pattern in swine was later confirmed by Rath *et al.* (1996), Rath *et al.* (1997). Cran *et al.* (1993) reported the birth of the IVF first calves using sperm sorted by flow cytometry. In a larger field study using IVF embryos, Cran *et al.* (1995) reported significantly altered sex ratios in dairy calves. However, Johnson *et al.* (1994) postulated that an increase in the rate of sperm sorting that would allow females to be inseminated artificially, would be needed in cattle industry. Catt *et al.* (1996) reported the birth of a male lamb following intracytoplasmic injection of a Y-sorted sperm. Ram spermatozoa, which have been sex-sorted exhibit higher motility, viability, acrosome integrity and mitochondrial activity than non-sorted (De Graaf *et al.* 2006; De Graaf *et al.* 2007c, Beilby *et al.* 2009). Johnson *et al.* (1989) reported the birth of the first offspring using flow cytometrically sorted sperm in rabbits. The sex ratio of the 37 offspring produced was similar to the sex ratio of the X- and Y-sorted sperm used for fertilization and was significantly altered from the expected sex ratio of 50% (6% and 81% male, respectively). Although sperm of all species can be sorted with high purity, achieving acceptable pregnancy rates using the low numbers of sperm needed for commercial application remains a major challenge in swine (Vasquez *et al.* 2009). Johnson (1991) has reported altered sex ratios in sows surgically inseminated with sexed sperm. Krueger (1999) and Krueger and Rath (2000) demonstrated that 50×10^6 sperm surgically deposited in close proximity to the utero-tubal junction were sufficient to obtain fertilization in swine. Currently, nonsurgical deep uterine insemination utilizing specially designed disposable catheters with a reduced number of sex-sorted sperm is possible (Martinez *et al.* 2001; Martinez *et al.* 2002; Grossfield *et al.* 2005). However, Rath *et al.* (2003) reported a reduced pregnancy rate and reduced litter size when employing this insemination method with sex-sorted sperm. The recent development of transgenic pigs with fluorophore-loaded spermatozoa (Garrels *et al.* 2011) may be instrumental for the establishment of direct comparative tests. In cattle,

Seidel *et al.* (1996) reported the birth of calves following artificial insemination (AI) of cows with an ultra-low number of sexed sperm. The results of this study were later confirmed by (Seidel *et al*. 1997; Seidel *et al*. 1998; Seidel *et al*. 1999). In all of these studies, the sex ratio of calves born from females inseminated with X- or Y-bearing sperm was significantly altered from the expected 50% sex ratio (~10% and ~90% male, respectively). The number of offspring produced utilizing sperm sexed by flow-cytometric sorting is estimated to be greater than 30000, with the majority of births occurring in cattle (Johnson *et al*. 2005; Katska-Ksiazkiewicz *et al*. 2006). However, limitations in the number of sperm cells that can be sexed in a given amount of time remains a major hurdle. Rens *et al.* (1998) reported the development of a novel nozzle that improves the efficiency of sexing sperm and has led to an increase in the number of cells that can be processed. In a modified protocol to process spermatozoa before, during and after sorting (Sexcess®; Rath *et al.* 2009b) the sample fluid is supplemented with Sodium fluoride (NaF). The presence of NaF in the sample fluid improves the post sort / post thaw maintenance of sperm motility in cattle (Klinc 2005; Rath *et al*. 2009a; Moench-Tegeder 2008; Moench-Tegeder 2011; Sun *et al.* 2010). Currently, sperm can be produced at a sorting rate of 15×10^6 sperm cells per hour (Johnson *et al*. 2005) and retain high-purity sorting of X- or Y-chromosome-bearing sperm can be achieved at rates up to 8000 cells per second for an input rate of 40000 X- and Y-sperms (Sharpe and Evans, 2009). In addition, a recent surge in the commercial production of IVF-derived cattle embryos is at least partially due to the use of flow-cytometrically, sex-sorted sperm (Wheeler *et al*. 2006). Recently, numerous publications on semen sexing using flow cytometry on other species are being reported in order to allow commercial use (Morris, 2005; O'Brien and Robeck, 2006; Karabinus, 2009; O'Brien *et al*. 2009; Rath *et al*. 2009b; Vasquez *et al*. 2009; Leahy *et al*. 2010; Gibb *et al*. 2011; Balao da Silva *et al*. 2012; Clulow *et al*. 2012). This ability to skew the sex ratio in the desired direction prior to fertilization and produce genetically superior offspring (Underwood *et al*. 2011) justifies the added expenses due to sorting cost and production inefficiencies.

Antigen H-Y

H-Y antigen is defined as a male histocompatibility antigen that causes rejection of male skin grafts by female recipients of the same inbred strain of rodents. It has been found on the surface of sperm (Hendriksen, 1999). Male-specific, or H-Y antigen (s), are also detected by cytotoxic T cells and antibodies. H-Y antigen appears to be an integral part of the membrane of most male cells. In addition, H-Y antibodies detect a soluble form of H-Y that is secreted by the testis. The gene (Smcy/SMCY) coding for H-Y antigen detected by T-cells has been cloned. It is expressed ubiquitously in male mice and humans and encodes an epitope that triggers a specific T-cell response *in vitro*. Additional epitopes coded for by different Y-chromosomal genes are probably required *in vivo* for the rejection of male grafts by female hosts. The molecular nature of H-Y antigen detected by antibodies on most male cells is not yet known. Testis-secreted, soluble H-Y antigen, however, was found to be identical to Müllerian-inhibiting substance (MIS). Identical findings were obtained for soluble H-Y antigen and MIS. Molecular data on this antigen or antigens are not yet available. Some studies have found that treating sperm with anti -H-Y antibodies prior to insemination slightly increased the number of females born (Zavos and Wilson, 1983). Studies that have looked directly for the H-Y antigen on sperm surfaces have had mixed results (Ali *et al*. 1990; 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).

Embryo sexing methods

X-linked enzyme activities

Embryos can be distinguished as male or female by measurement of the gene dosage for X-linked enzymes. Two laboratories have reported the sexing of mouse embryos by this principle, one by measuring the activity of hypoxanthine phosphoribosyl transferase (HPRT; Monk and Handyside, 1988) and the other by measuring glucose 6-phosphate dehydrogenase (G6PD) activity. In the latter experiment, d-4 mouse embryos were assayed directly for G6PD activity. The population of embryos showed a bimodal distribution for G6PD activity, but overall only 64% of the pups born were correctly sexed. Embryo viability was reduced, particularly for embryos with very high or very low enzyme activity. In comparison with embryos derived from unsorted spermatozoa, bovine and ovine IVF-embryos derived from sex-sorted spermatozoa display a reduction in the relative abundance of developmentally important genes like Glucose transporter 3 Glut3 and G6PD (Morton *et al*. 2007; Beilby *et al.* 2011), which may be deleterious to the developmental competence of embryos. Monk and Handyside (1988) removed a single blastomere from 8-cell mouse embryos for assay of HPRT activity and the remainder of the embryo was not exposed to the potentially toxic components of the assay. These authors simultaneously measured the activity of the autosomal linked enzyme, adenine phosphoribosyl transferase (APRTI), which provided a control for differences in overall enzymatic activities between embryos. In this case, sex determination was done by calculating the ratio of HPRT to APRT activities. Although the

distribution of this ratio was clearly bimodal, a number of embryos had intermediate values and it was not clear how these embryos were classified. Because not all the embryos assayed were transferred, it was not possible to determine whether the embryos with intermediate values were accurately sexed. Fourteen of the 15 fetuses obtained from sexed embryos were of the sex predicted. This approach to embryo sexing is complicated by the fact that variable X-chromosome dosage is limited to the period after the activation of the embryonic genome and before X-chromosome inactivation. Activation of the embryonic genome in bovine embryos occurs between the 8- and 16-cell stages (Frei *et al*. 1989); therefore, if 8-cell bovine embryos were assayed as described by Monk and Handyside (1988), it is unlikely that a bimodal distribution of enzymatic activity would be observed. The exact timing of X-chromosome inactivation in embryos of domestic animals is not known, but it likely begins to occur during the blastocyst stage (Chapman, 1985). For the embryonic stages most commonly manipulated in the commercial embryo transfer industry, this could very well lead to diagnosis of female embryos as male due to early X-chromosome inactivation or to ambiguous results due to partial X-chromosome inactivation.

PCR amplification

Several Y-chromosome specific DNA probes have been reported (Leonard *et al*. 1987; Matthews *et al*. 1987; Bondioli *et al*. 1989). Matthews *et al*. (1987) claim that their assay can detect Y-linked DNA repeats in a single Y-chromosome within 3 h. When applied in the field, these methods of embryo sexing have proved to be 95-100% accurate. Bondioli *et al*. (1989) reported over 40% pregnancies from frozen-thawed embryos which had been biopsied for sex determination prior to freezing. PCR-based sexing assays are generally favoured, because of the advantages of being relatively simple, rapid, and inexpensive. The first demonstrated sexing of goat embryo with PCR amplified DNA from blood sample was in 1990 (Aasen and Medrano, 1990). Subsequently, an accurate, reliable and rapid PCR method had been standardized for accurate sex determination in goats (Rao and Totey, 1992). Leoni *et al*. (1996) first described a method for sex determination in goat embryos, using PCR and restriction fragment length polymorphism (RFLP) analysis. They amplified a DNA fragment derived from four to eight cells that had been biopsied from embryos described by Aasen and Medrano (1990). However, the risks would be increased in contamination and misdiagnosis, because of limited amount of DNA in embryo biopsies, cross-species contamination (Aasen and Medrano, 1990; Gutiérrez-Adán *et al*. 1996) and requirement more time to specific endonuclease digestion for RFLP analysis (Aasen and Medrano, 1990). Therefore, improvement of embryo-based techniques is essential in this species (Chen *et al*. 2007). The amelogenin gene, which exists on both X- and Y-chromosomes (AMELX and AMELY), encodes an important protein in the developing mammalian tooth and enamel matrix that has been conserved during the evolution of vertebrates. Several studies have showed the amelogenin amplification by PCR is a reliable method for sex determination in cattle (Chen *et al*. 1999), sheep and deer (Pfeiffer and Brening, 2005; Dervishi *et al*. 2008), goats (Chang *et al*. 2006; Weikard *et al*. 2006; Malik *et al*. 2013) as well as in the related species (Weikard *et al*. 2006). The use of this gene has made the sex determination much less complicated, since only pair of primers is required to amplify the different size fragments of the amelogenin genes (Chen *et al*. 1999; Weikard *et al*. 2006). It is well known that sex and satellite chromosome specific sequences are highly conserved in the Bovidae family during evolution, allowing the use of heterologous PCR primer pairs in closely related species (Moore *et al*. 1991; Mara *et al*. 2004). Several protocols made use of bovine Y-chromosome sequence derived primers on sheep blood cell DNA; trophoblastic cells detect the sex of sheep embryos (Rao and Totey, 1992; Mara *et al*. 2004). The conserved status of the amelogenin gene among vertebrates indicates the possibility to use the test in cattle, sheep, red deer, and other mammal species (Pfeiffer and Brening, 2005). Better results were published when the PCR amplification of specific DNA sequences was used to determine the embryonic sex in cattle (Herr *et al*. 1990; Schroder *et al*. 1990; Peura *et al*. 1991), pigs (Pomp *et al*. 1995), horses (Peippo *et al*. 1995) and mice (Han *et al*. 1993). The recently published results support the efficiency of PCR method for sex determination in cattle with a high accuracy and in an acceptable time intervals (Thibier and Nibart, 1995; Lopes *et al*. 2001; Ekici *et al*. 2006; Yu *et al*. 2006). Flushed embryos from superovulated donors are almost exclusively used for the determination of sex in farming conditions.

Male-specific antigens

Using immunological techniques, the presence of H-Y antigen has been demonstrated on cells of 8-cell or later stage bovine (White *et al*. 1987a), porcine (White *et al*. 1985) and ovine (White *et al*. 1987b) embryos. Antisera to H-Y antigen have typically been prepared by injection of spleen cells from male mice into females of the same strain. Response to this immunization is unpredictable; thus, sera must be screened for anti-H-Y activity. The most common method used to identify high-titer sera has been the sperm cytotoxicity assay (Goldberg *et al*. 1971; Piedrahita and Anderson, 1985). Initial attempts to use these antisera to sex embryos also employed a cytotoxicity assay. Following exposure to H-Y antiserum and complement, embryos are

classified as "affected" or male, when cell lysis or failure to develop in culture is observed. "Unaffected" embryos are classified as female and are available for transfer (Shelton and Goldberg, 1984; Anderson, 1987). Embryo cytotoxicity assays are limited to the production of female offspring because male embryos are destroyed. This limitation was overcome in the research of Utsumi *et al.* (1984). In these experiments, embryos were incubated with antibodies to rat H-Y antigen in the absence of complement. This incubation tended to retard further development of male embryos but not to affect female embryos. Upon removal of the antibody, both groups proceeded to develop, allowing transfer of both male and female selected embryos. Indirect immunofluorescence assays have been developed using polyclonal and monoclonal antibodies to H-Y antigen with bovine (White *et al*. 1984, White *et al*.1987a; Wachtel *et al.* 1988; Booman *et al*. 1989), porcine (White *et al*. 1985) and ovine (White *et al*. 1987b) embryos. In these assays, embryos are typically incubated with the primary H-Y antibody for 30 to 40 min, carried through several washes and incubated with a secondary antibody, usually labeled with fluorescein isothiocyanate (FITC). In one study (Booman *et al*. 1989), the FITC label was replaced with R- phycoerythrin (RPE) in an attempt to increase the intensity of the fluorescence signal. Despite numerous attempts to optimize these assays, the accuracy of sex prediction has not been any higher than the accuracy described above for the cytotoxicity assays (Booman *et al*. 1989). Several reasons have been noted for this apparent inability to increase the accuracy of this approach.

Antibodies against H-Y antigen are not entirely sex-specific; cross-reactions of these antibodies (both polyclonal and monoclonal) will occur (Wachtel, 1983), resulting in false positives. Antibodies to H-Y antigen are typically low-affinity and the H-Y antigen is sparsely expressed on embryonic cells, producing weak and highly subjective fluorescent signals. Increasing signal intensity decreased the subjectivity of the assay, but accuracy of sex prediction was not improved (Booman *et al*. 1989). The problem of weak positive signals is complicated by the existence of nonspecific fluorescence. Lysed or dead extruded blastomeres (common in bovine embryos) usually exhibit fluorescence; fluorescence in the zona pellucida is sometimes observed, and diffuse fluorescence in the perivitelline space is common (Booman *et al*. 1989).

The advantages of an immunological approach to embryo sexing are considerable. The procedures are noninvasive and require no special manipulation skills and embryo viability apparently is not compromised. Indirect immunofluorescence techniques can be completed quickly, allowing embryos to be sexed prior to transfer without cryopreservation. The enzyme labels who can replace the fluorescence labels are not yet available to allow embryos to be sexed in the field.

Cytological methods

Identification of the Barr body has been used to sex rabbit embryos, but in the embryos of most domestic species the granular nature of the cytoplasm makes it difficult to see the Barr body (Rowson, 1974). Hare *et al.* (1976) described the sexing of embryos by karyotyping trophoblast biopsies, and Wintenberger-Torres and Popescu (1980) sexed 60% of 149 blastocysts by karyotype. The limited number of cells in metaphase prevented a certain diagnosis in some embryos. Moustafa *et al.* (1978) reported a method in which a small number of cells were removed from 6-day embryos for karyotyping, but Singh and Hare (1980) found that only 33% of the embryos could be sexed by this method. Picard *et al.* (1984) were able to sex 60% of the embryos by bisecting the embryo and culturing one half for 4 h. Further development of this method is needed to improve its efficiency and to enhance survival of the remaining half embryo, particularly when frozen.

***In vitro* maturation (IVM), *in vitro* fertilization (IVF) and sex selection**

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. When bovine oocytes were inseminated immediately after maturation, more females were detected; in contrast, when insemination was delayed more males were produced (Gutierrez - Adan *et al*. 1999). It was suggested that these differences may have been due to the oocyte which having differing ability to process X and Y sperm depending on its maturational status, 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 favoured, 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. There are also indications that culture conditions could account for the disparity between sexes in speed of development (Marquant-le-Guienne and Humblot, 1998;

Kochhar *et al*. 2001; Lonergan *et al*. 2001). Components of the culture media could favor sex differences based on developmental speed, survival or both, due to metabolic differences (Yadav *et al*. 1993; Grisart *et al*. 1995). In that regard, glucose, which seems to increase the developmental speed of male embryos (Peippo and Bredbacka, 1996; Bredbacka and Bredbacka, 1996) is frequently present in embryo culture media (Takahashi and First, 1992). Rheingantz *et al*. (2003) and Rheingantz *et al*. (2006) reported that the swim-up method of sperm separation created a deviation in the sex ratio, resulting in a significantly higher proportion of male embryos among the most-developed embryos (Kobayashi *et al*. 2004; Madrid-Bury *et al*. 2003). Moreover, the swim-up method resulted in a higher rate of male embryos than the Percoll gradient method across all embryos produced. In contrast, the Percoll gradient method did not alter the sex ratio across all embryos. 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. Following sperm and embryo sexing techniques developed in the laboratory, sex can also be selected in the field with artificial insemination, embryo transfer and factors such as asymmetric distribution within the uterus.

Type and timing of insemination

Variation in the timing of insemination relative to ovulation has been shown to be related to the sex ratio (Maramatsu and Kawanishi, 1975; Sales *et al*. 2011; Kharche *et al*. 2013). The size of the follicle from which ovulation occurs and the occurrence of estrus from progesterone (P4) source removal to the timed AI (TAI) have been reported to influence pregnancy per AI (Perry *et al*. 2005; Perry *et al*. 2007; Sá Filho *et al*. 2010b; Sá Filho *et al*. 2011; Neves, 2010). In suckled *Bos indicus* cows presenting a larger follicle (≥9 mm), pregnancy per AI was similar when using either sex-sorted or non-sex-sorted sperm. In *Bos indicus* cows, a positive effect of increased largest follicle diameter at TAI on ovulation and pregnancy per AI using non-sex-sorted sperm has also been verified (Meneghetti *et al*. 2009; Sá Filho *et al*. 2010b). With sex-sorted sperm, increased pregnancy per AI can be reached if the AI is performed closer to ovulation (Schenk *et al*. 2009; Sá Filho *et al*. 2010a; Sales *et al*. 2011). Furthermore, the sex ratio is affected by the age or size of the inseminated mother. It was assumed that the age of the mother would be related to condition, such as older cows in poorer condition than younger cows (Saltz, 2001; Saltz and Kotler, 2003). An increase in maternal age, however, may also be associated with the increased production of sons, leading to a male-skewed sex ratio; this would be because of an increased social status in females in relation to their age or size (Cameron *et al*. 2000; Côté and Festa-Bianchet, 2001; Vissche *et al*. 2004).

Embryo transfer and sex ratio

Known sex of embryos in embryo transfer (ET) programs can more effectively help to manage producer resources because more heifer calves per ET can be produced. While the overall sex ratio of calves produced from embryo transfer has been reported to be slightly higher than parity (Thompson, 1997; Kochhar *et al*. 2001; Larson *et al*. 2001; Peippo *et al*. 2001), the sex ratio of calves resulting from transfers to specific uterine horns remains unknown. An altered sex ratio from the left and right uterine horns may indicate uterine selection pressures providing a preferential advantage to embryos of one sex. Transuterine migration occurs with high frequency in litter producing species such as the pig (Pope *et al*. 1986), as well as in species that regularly produce twins and higher order births, such as sheep (Doney *et al*. 1973) and goats (Mani *et al*. 1992). In heifer, Hylan (2002) and Hylan (2007) reported that significantly more calves were produced from right horn transfers than from left horn transfers. Previous studies have indicated that cattle ovulate from the right ovary more than from the left ovary (Scanlon, 1972; DelCampo *et al*. 1977). However, in a study of slaughter house reproductive tracts, Hylan (2007) indicated that there is no preferential selection for embryos of a single sex in the uterine horns of recipient cattle. In addition ovary of origin from which the pregnancy is derived, rather than the uterine horn of gestation, may influence the sex of offspring in cattle. In cattle, this sex-dependent developmental asynchrony has been described in both *in vivo* (Gutierrez-Adan *et al*. 1999) and *in vitro*-derived (Xu *et al*. 1992; Tominaga *et al*. 1996) embryos. Hylan (2007) indicated that asynchronous development failure may be due to inferior oocyte maturational quality at the time of fertilization. Callesen *et al*. (1986) described an increase in abnormal follicle development, while Callesen *et al*. (1987) reported an increase in immature oocyte ovulations in superovulated cattle. In addition, an associated decrease in embryo quality was also described in both reports. It is generally accepted that high-quality embryos are the most suitable for sex determination. However, the flushings from superovulated donors usually contain embryos of various categories and often, after evaluation under the stereomicroscope, only a limited number of embryos meets criteria typical of high-quality embryos. The use of lower-quality embryos is also possible by the isolation of blastomeres extruded into the perivitelline space (Yu *et al*. 2006; Lopatarova *et al*. 2007). This approach enlarges the spectrum of embryos which could be employed for the successful sex determination. However, the completed sex determination is lower. The splitting of

excellent or good embryos with following biopsy is another possibility of increasing the pregnancy rates of desired sex from one superovulation. Commercial ET programs using the splitting technology have reported pregnancy rates ranging from 100 to 113% after single and 84% after double demi-embryo transfer to each recipient (Lopes *et al*. 2001).

Distribution of sexes within the uterine horns

Asymmetric distribution of the sexes within the uterus of pregnant mammals has been described (Andersson *et al*. 2004; Kurykin *et al*. 2007). In Mongolian gerbil, an excess of male fetuses in the right horn has been observed (Clark *et al*. 1991). In contrast, Clark *et al*. (1991) failed to detect any sexual segregation within the uterine horns in the mouse. Herbert and Bruce (1980) also failed to find a statistical difference in the sex ratio between the left and right uterine horns in the rat. The partial segregation of sexes observed in the uterus of the gerbil, rabbit and mouse suggest some consistent lateral asymmetry either between the left and right uterine horns or the left and right ovaries in these species. In rats, the left uterine horn contains fewer implantation sites than the right horn (Buchanan, 1974). A larger number of embryos are gestated in the right uterine horn in mice (Wiebold and Becker, 1987) and in hamsters, a greater number of sperm are present in the right uterine horn after mating (O and Chow, 1987). In human fetuses, the right ovary is larger than the left (Mittwoch and Kirk, 1975) and in hamsters, mice and rats the right ovary contains more corpora lutea (Long *et al*. 1991; Fritzsche *et al*. 2000). In pigs (Hunter *et al*. 1985) testes or ovotestes in hermaphrodites occur predominantly on the right side. In contrast, however, testes or ovotestes occur predominantly on the left in the mouse (Ward *et al*. 1987). In mare Arthur (1958) described ovarian activity and noted a greater proportion of corpora lutea (CLs) present on the left ovary compared with the right ovary. Casida *et al*. (1966) reported that the right ovary in sheep produces more corpora lutea than the left ovary. Similarly, in the goat, Lyngset (1968) found that the right ovary was more active than the left ovary, having a greater number of large follicles. James (1982) examined the sexes of piglets within the uterine horns of sows and noted that the sexes were not associated with the side of the uterine horn in which they were gestated. Numerous studies have demonstrated lateral asymmetries in the cow. Scanlon (1972) reported more ovulations from the right ovary compared with the left ovary. Hylan *et al*. (2002) and Hylan (2007) demonstrated that, in heifers and cows, the sex ratios of calves gestated in the left and right uterine horns are significantly different ($P<0.001$) and are also different from parity ($P<0.001$). However, given the altered sex ratios detected in the left and right uterine horns in normal females and in ovarian translocated individuals, the probability of transuterine migration occurring in this species appears to be relatively low. Hylan (2007) indicated in her experiment that the ovary of origin of the oocyte may also influence the sex of the offspring in cows. However, even though transuterine migration has been reported as an extremely infrequent occurrence in cattle (0 to 3%) (Scanlon, 1972), the same conclusion cannot be reached in cattle. Because the number of matings prior to the pregnancies investigated in this experiment, and the sex of any possible pregnancies remains unknown, sex-specific embryonic mortality within individual uterine horns to achieve a pregnancy of the preferential sex remains a possibility. Trivers and Willard (1973) originally suggested that alteration of the sex ratio might be accomplished through parental manipulation of postnatal mortality, suggesting that mothers should prematurely terminate investment in offspring that were less likely to breed successfully or fail to achieve their maximum reproductive potential. Likewise, manipulation of the sex ratio before birth, as hypothesized by Maynard-Smith (1980), would be preferential and would minimize reproductive inefficiencies. Further investigations into the underlying mechanisms which are responsible for the skewed sex ratios in the left and right uterine horns are needed. Assisted reproductive technologies such as AI, IVF and ET, as well as advanced biotechnologies like PCR, can provide insight into these mechanisms in a more time-efficient manner.

CONCLUSION

Several methods of predetermined sex can be altered. Serum albumin gradient swim-up and flow cytometry have been used repeatedly for sexing sperm. The amelogenin amplification by PCR is presented as a reliable method for sex determination in farm species embryos. Still, control of these methods is much less than perfect as revealed by the variations in response between animals, localities, years, genotypes, AI and ET techniques. Because results do not easily predict with accuracy, there will be continuing incentive to improve these techniques.

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