—Mini Review—

# Factors Affecting the Primary Sex Ratio in Cows

*Hisataka Iwata*

*Tokyo University of Agriculture, Kanagawa 243-0037, Japan*

***Abstract:*** *In the dairy and beef cattle industries, artificial insemination and embryo transfer are common. Many studies have demonstrated a skewed sex ratio in the calves derived from assisted reproductive technologies. During the period between insemination and calving, insemination condition and the environment in which embryo development occurs are believed to affect the sex of an embryo. This short review looks at studies of factors that have been reported to affect the primary sex ratio of bovine embryos, including the timing of insemination, sperm and oocyte status, and maternal conditions.*

***Key words:*** *Primary sex ratio, Cow, Insemination, In vitro fertilization*

## Introduction

In the dairy industry, milk production is the monopoly of females, but in the beef industry, the rate of return on investment is much greater for males than for females; thus the sex ratio of calves is a crucial factor in both the industries. In general, sex at birth does not necessarily reflect the sex of the zygote; the primary sex ratio is determined when X or Y chromosome-bearing sperms fertilize an oocyte, whereas the secondary sex ratio is based on sex at birth. Between fertilization and birth, it is assumed that there are many factors that affect the sex ratio of an embryo, including differential motility and transport to the oocyte between X and Y chromosome-bearing sperms, selective advantages in binding, penetrating and fertilizing the oocytes for X and Y chromosome-bearing sperm, differential developmental competence of male and female embryos, and selective male or female embryo loss during pre-implantation and pregnancy [1]. In this article, I introduce the factors that are assumed to affect the primary sex ratio in cows. First, I discuss studies relating to the effect of the timing of insemination on the sex of offsprings in artificial insemination (AI). Such studies, it is difficult to determine the primary sex ratio in living organisms, and the methods used inevitably involve factors that are known to affect the secondary sex ratio. I then discuss the studies which have reported the factors affecting the primary sex ratio of embryos produced *in vitro*. These factors include sperm and oocyte co-incubation periods, sperm and oocyte status, and maternal conditions.


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e-mail: h1iwata@nodai.ac.jp

## Timing of AI Service

The efficiency of AI depends on the timing of insemination. Farmers have a vested interest not only in the outcome of AI but also in the sex of the offspring. Based on their experience, many farmers assume that early insemination favors female offspring, whereas late insemination favors male offspring. The terms early and late AI are determined on the basis of the timing of AI relative to the timing of ovulation, which is detected by ultrasonography or predicted by the timing of either the onset of estrus or hormonal treatment. In the field, estrus of cows is detected by visual inspection and/or by using devices for detection of mounting behavior, or for the determination of vaginal–cervical mucus conductivity. Wehner *et al.* [2] reported that vaginal–cervical mucus conductivity readings closely reflected the onset and end of estrus and that performing AI at the time when the conductivity declined (ie., at the onset of estrus) resulted in 13 females out of a total of 14 calves, whereas AI performed when the conductivity rebounded resulted in 11 males out of a total of 12 calves. In contrast, the sex of the calves derived from natural mating or normal estrus AI did not differ from the expected 1:1 ratio. Martinez *et al.* [3] conducted 716 AI experiments at 8–18, 8–30, or ≥30 h after visual detection of the onset of estrus, and reported that delayed AI (performed at ≥30 h) significantly skewed the sex ratio in favor of male calves (72.1%). Similar results were reported for ewes: those served early relative to the

timing of ovulation (5 h before) favored females and late service (5 h after) favored males [4]. Martinez *et al.* also examined the effect of the timing of insemination on the sex ratio of embryos produced *in vitro*(8-cell stage), and reported that oocytes fertilized immediately after polar body extrusion gave in a greater population of female embryos, whereas prolonging the maturation period favored male embryos. They suggested that the difference in the ability of X or Y chromosome-bearing sperm to fertilize oocytes depended either on the time of insemination or on the oocyte maturation status.

On the other hand, there have been contrary reports to these results in the literature. For example, Rorie *et al*. [5] synchronized the ovulation of 98 cows and 41 heifers with GnRH administration and followed it 7 days later with PGF2α administration. Onset of estrus was detected by mounting activity, and insemination was performed at either 8–10 h or 20–25 h after the onset of estrus, and the sex of the fetus was determined by ultrasonography. They reported that there was no significant bias in the sex ratio. In addition, Roelofs *et al*. [6] reported that when the ovulation of cows was observed by repeated ultrasonography and AI was performed from 36 h before to 12 h after ovulation, the male/female ratio of day-7 embryos was not affected by the timing of insemination. On the other hand, Pursley *et al*. [7] synchronized the ovulation of cows with GnRH administration, followed it 7 days later with PGF2α administration, and 2 days thereafter with a second GnRH treatment. The cows were inseminated at 0, 8, 24, and 32 h after the second GnRH treatment resulting in a U shaped ratio of female offspring with a higher percentage of males when AI was performed before and after ovulation and a lower percentage near the time of ovulation. The major reason for these conflicting results is the complexities of the multiple factors. Estrus length and the timing of ovulation differ greatly among individual cows. It is also difficult to determine the precise timing of ovulation and the primary sex of the resultant embryos present in the oviductal tube. In addition, semen and bulls, heifer or cows, and synchronization of ovulation itself reportedly affect the sex of the offspring [8–11]. Moreover, the intrinsic developmental competence of embryos in some environments differs between genders. There is evidence that culture conditions for early embryo development can skew the sex ratio of the embryos. In cows, early embryo development is faster for male embryos than for female embryos [12, 13]. Glucose metabolism and mitochondrial DNA copy number in bovine embryos [14–16] are greater among male embryos than amoung female embryos, and a high glucose concentration has been shown to be detrimental to female embryos [17]. However, female embryos have a greater tolerance to reactive oxygen-derived stress [18, 19]. These differences are believed to be caused by differential expression of either X chromosome-linked genes or different epigenetics between embryos of the two genders [20, 21]. Moreover, the oviductal and uterine environments are inevitably affected by maternal physiological conditions, which affect both the primary and secondary sex ratio (see the section below).

## IVF

*In vitro* fertilization (IVF) is, to a certain extent, a useful tool for elucidating the causes of skewed sex ratios derived from the timing of AI. There have been many studies on the factors that affect the sex ratio of bovine embryos produced *in vitro*. Some of these demonstrated that the sex ratio of such embryos is skewed in favor of male embryos [22, 23], and this difference has been detected as early as the 2-cell stage [24]. It is assumed that there are some selective advantages for X or Y chromosome bearing sperm in IVF conditions, such as sperm longevity and capacitation, sperm-oocyte interactions, and oocyte status.

### Length of Sperm-oocyte Co-incubation

The first factor adduced as a candidate affecting the sex ratio of bovine embryos is the length of the sperm-oocyte co-incubation period. Kochher *et al*. [25] reported that when cumulus cells and oocyte complexes (COCs) were co-incubated with sperm for 6 or 18 h, the rate of fertilization was decreased in the shorter incubation period, but the male/female ratio was approximately 3.6:1 in 8-cell stage embryos and 2.1:1 in blastocysts, whereas the ratio for the longer co-incubation period (18 h) was 1:1. Moreover, Iwata *et al*. [26] reported that a short period of sperm-COCs co-incubation also skewed the sex ratio of the 8-cell stage embryos in favor of male embryos, but in longer co-incubation, the skewed sex ratio reversed to 1:1. They reported that this trend was also observed in co-incubation experiment with sperm-denuded oocytes. These results indicate that Y chromosome-bearing sperm have a selective advantage in primary sperm-oocyte interactions. In this context, we co-incubated bovine sperm and zona pellucida (ZPe) derived from oocyte matured *in vitro* for short and long periods, and determined the ratio of Y chromosome-bearing sperm among the sperms attached to the ZPe using *in situ* hybridization. The ratio of Y chromosome-bearing sperm was significantly greater than the expected ratio of 1:1 for

the shorter incubation period (55.1%), and in the longer co-incubation period, the ratio of Y chromosome-bearing sperm was reversed (49.2%) [27]. Indeed, the primary sperm-ZPe attachment is not necessarily related to successful fertilization, and it is unclear whether the ratio of Y chromosome-bearing sperm reflects the primary sex ratio; hence, further experiments are required to clarify this issue. However, as described above, there is a clear difference in the fertilization processes between X and Y chromosome-bearing sperm.

## Characteristics of the X and Y Chromosome-bearing Sperm

Sperm ejaculated into the female reproductive tract has to undergo physiological changes prior to fertilization. The uterine, utero-tubal junction, and the oviduct have discrete roles in selecting functional sperm [28]. On entering the oviduct, sperm get attached to the epithelial cells of the isthmus till ovulation. Once capacitation occurs, sperm escape from the isthmus reservoir thorough hyperactivated motility and move toward the oocyte in the ampullary region [29]. Thus, the effect of sperm pre-incubation prior to fertilization on the sex of the resultant embryo has been studied on the premise that the timing of capacitation between Y and X chromosome-bearing sperm differs. Lechniak *et al*. [24] reported that 24 h of pre-incubation of bovine sperm resulted in a significantly greater ratio of females among hatched blastocysts. Hendricks *et al*. [30] reported that 4 h of pre-incubation at 38.5°C significantly increased the ratio of female blastocysts compared to that of blastocysts derived from the same IVF procedure without sperm pre-incubation. Pre-incubation of bovine sperm induces capacitation, which increases sperm-ZPe binding and sperm motility. In addition, 6 h of sperm-oocyte co-incubation in the same study resulted in a ratio of 67.3% (74/114) male 8-cell stage embryos, and this ratio is significantly different from the expected ratio of 1:1. However, pre-incubation of sperm prior to fertilization decreased the ratio to 56.4% (62/112) [26]. These reports suggest that there are some properties of Y chromosome-bearing sperm that act preferentially during fertilization.

For decades, it has been suggested that X and Y chromosome-bearing sperm can be separated on the basis of motility [31]. In cows, Madrid-Bury *et al*. [32] showed that a routine swim-up procedure cannot isolate motile sperm fractions with a sex ratio distortion, but their modified swim-up technique using heparin resulted in a high percentage of X chromosome-bearing sperm. They suggested that the different physiological activity between X and Y chromosome-bearing sperm is based on the different timing of capacitation/acrosome reaction. Ethical considerations make it difficult to study the properties of X and Y chromosome-bearing sperm of humans, but in cows, sex-sorted sperm have been widely used over the past decade, and this situation has encouraged the study of the characteristics of X and Y chromosome-bearing sperm. Bermejo-Alvarez P *et al*. [33] demonstrated that when bovine embryos were produced *in vitro*, there was no difference in the rate of cleavage and blastulation between X and Y chromosome-bearing sorted sperm, although the rate was significantly lower than that of unsorted sperm. At the time of writing, there were no reports of differences in the inducibility of capacitation, the acrosome reaction, or penetration between sorted X and Y chromosome-bearing sperm.

## Oocyte Conditions and Length of Maturation

In cows, when full grown oocytes are harvested from antral follicles and cultured *in vitro*, meiotic resumption occurs immediately and the oocytes reach the metaphase 2 stage within 18–24 h. In order to achieve a successful fertilization, it is important to determine the optimal maturation period, but prolonging the maturation period results in cellular and molecular changes in oocytes and decreases the developmental competence [34]. Agung *et al*. [35] cultured bovine oocytes for 16, 22, 28, and 36 h, and then co-incubated these oocytes with sperm for 5 h. The rate of blastulation was highest for the 22 h maturation period, and the ratio of male blastocysts was lowest for the oocytes that were matured for 16 h (31.8%), and highest for those matured for 36 h (72.7%). The trend that fast fertilization relative to the timing of oocyte maturation favors female embryos and late fertilization favors male embryos was also demonstrated by Gutiérrez-Adán *et al* [4]. Moreover, Dominko and First [36] cultured bovine oocytes for 16 h and then removed the cumulus cells. When the oocytes were fertilized immediately after selection for the presence of the first polar body, the male/female ratio of the resultant 2-cell stage embryos was 0.5:1, whereas fertilization 8 h after the selection on the basis of polar body extrusion increased the male/female ratio to 2.2:1. Dominko and First suggested that amount of sex chromosome-linked transcription may differ between X and Y chromosome-bearing sperm based on the premise that sperm DNA is transcribed before as well as after completion of meiotic division [37]. Oocytes of different maturities have different abilities to process these transcripts. In addition, we demonstrated that during *in vitro* maturation of porcine

oocytes, ZPe interactions with sperm increase during oocyte maturation and decrease once the oocytes begin aging [38]. Rath *et al*. [39] reported that the ZPe undergoes biochemical changes during porcine oocyte maturation prior to fertilization, although there was no tangible evidence of whether the changes in the ZPe preferentially affected interactions between the ZPe and X or Y chromosome-bearing sperm. There is, of course, controversy over the assumption that oocyte maturation length affects the sex ratio of the resultant embryos. Rizos *et al*. [40] cultured oocytes for 16 and 24 h and fertilized them without performing polar body selection. When the sex of all of the 853 2-cell stage embryos and 458 blastocysts was determined, they concluded that the overall sex ratio of all 2-cell stage embryos was skewed toward males, irrespective of the maturation period, and they suggested that the maturation period does not affect the sex ratio of the embryos. In contrast, Park *et al*. [41] demonstrated antithetical results when they produced embryos derived from oocytes that were matured for 18 h or 24 h *in vitro* and transferred to cows. The sex ratio of the calves was similar between the *in vivo* group and the 24 h group, whilst the calves produced from embryos derived from the 18 h group were all males, although, the number of calves examined was small.

## Maternal Conditions

It has also been suggested that the maternal condition during insemination and pregnancy is a significant factor affecting the sex of the offspring [42]. High maternal dominance rank has been shown to be associated with an increased ratio of male offspring in red deer [43], swine [44], and primates [45]. On the other hand, as maternal condition declines, more females are produced [46]. It has also been shown that chronic exposure to severe stress increases the ratio of male offspring, whereas shorter exposure to stress increases the ratio of female offspring in humans [47]. In cows, when follicular phase cows are exposed to stress, a higher percentage of embryos collected after superovulation are female [48]. Dominance is a behavioral trait that is influenced by testosterone in mammals [49, 50] and the maternal testosterone level probably affects the sex ratio of offspring [51]. In addition, treating cows with testosterone encourages their resistance to unfamiliar environments [52], and heat stress increases the estradiol/testosterone ratio in follicular fluid [53]. Grant *et al.* [54] collected ovaries from a slaughterhouse and examined the concentration of testosterone in the follicular fluid as well as the sex of the 6–8 cell stage embryos derived from the oocytes enclosed in the follicles. They reported that when the testosterone concentration in the follicular fluid was high, the enclosed oocyte was more likely to produce male embryos. Furthermore, Grant *et al*. [55] showed that the concentration of testosterone in the follicular fluid of subordinate follicles was significantly related to the production of male embryos, but the concentration of estradiol was not. Contrary to the hypothesis that testosterone or stress conditions may affect the sex ratio of bovine embryos, Díez *et al.* [56] collected bovine oocytes and cultured them for 22 h in medium containing roscovitine, a cyclin-dependent kinase inhibitor, and then subjected them to *in vitro* maturation. Neither supplementation of these media with testosterone nor high temperature stress during the culture period affected the sex ratio of the resultant embryos. Testosterone in follicular fluid is a crucial factor affecting oocyte development and maturation [57–59], and a relationship between testosterone and glucose concentrations in blood has been reported [41]. Dominance of the mother is closely related to better nutrition, and a better body condition has been shown to be related to preferential male progeny in many species [1]. More than 30 years ago, Skjervold *et al*. [60] reported that dairy cows with high levels of nutrition produced more male calves than those on a poor diet. In addition, circulating glucose is related to maternal nutrition and stress [41]. Moreover, as described above, glucose concentration affects the early development of bovine embryos. These findings suggest that the conditions of the oocyte donor should be consider when examining factors affecting the primary sex ratio.

## Perspective

In this review, I have introduced studies which have investigated factors affecting the primary sex ratio of bovine embryos. All of these factors are controversial, and all the previous studies used complex methods and experimental conditions that were subsequently suggested as being among the many factors affecting the sex of an embryo. Further studies are therefore required to construct a coherent theory. The cattle industry is a major user of assisted reproductive technologies involving *in vitro* production of embryos and sexing of the embryo. Novel technologies for obtaining sex-sorted semen and for ovum pick-up followed by IVF are becoming popular. Under these conditions, it should be relatively easy to tackle the enigmatic factors associated with the biased sex ratio of calves, and information obtained in the field as well as in the laboratory may help our understanding of sex biasing factors in humans.

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