Advances in sex ratio management in cattle

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Abstract

Manipulation of the sex ratio of cattle offspring can be documented as far back as 1891 when Russell analyzed the relationship between the sex of a calf and the time during estrus at which copulation occurred.[1] Interestingly, or perhaps embarrassingly, researchers have made little progress in the advancement of sex ratio manipulation, although there have been copious attempts in the last century. Most methods of manipulating embryo sex attempt to exploit the differences between X- and Y- bearing sperm. Mass and surface proteins have been predicted to differ between X- and Y- bearing sperm and may provide an approach for separation through techniques such as flow cytometry, sperm surface antigen binding, and albumin separation. Other studies have discovered correlations between pharmacological influence, semen incubation, the timing of insemination relative to ovulation, and maternal physiological influence on the sex of offspring. Still other observations included the effects of acute stressors, drugs, environment, and paternal occupation on the sex ratio of offspring. The purpose of this review is to provide an update on existing sex selection technologies as well as present novel sex selection data in beef cattle.

Keywords: Cattle, sex ratio, sperm

Introduction

Swaying the sex ratio is beneficial in agriculture and human fertility medicine. In the dairy industry, female calves are significantly more valuable because of their ability to produce milk, whereas in beef production, male calves are more desirable due to their rapid growth and efficient meat production. In the swine industry, males are undesirable due to boar taint, an objectionable flavor and odor associated with the meat of intact males. Further, gilts reach market weight quicker than males thereby decreasing overall feed costs. Niche markets, such as endangered species, pets, or laboratory animals may also prove to be useful applications for sex selection.[2] To date, there is no effective, practical, inexpensive method for manipulating the sex of offspring.

Sex determination

In mammals, somatic sex of the offspring is determined by the presence or absence of the Y-chromosome. An XX genotype results in a female while an XY genotype produces a male.[2] The haploid oocyte from the female always contains an X- chromosome, whereas males produce both X- and Y-bearing haploid sperm, governing which sex will result upon fertilization of the oocyte. It is a common estimation that in most mammalian species, males produce X- and Y- bearing sperm in a 1:1 ratio, resulting in male or female embryos of the same proportion.

SRY gene

The sex-determining region of the Y- chromosome, or SRY gene, was discovered in 1990 and is located near the tip of the chromosome's short arm.[4] It is an intronless region that encodes for a nuclear factor-like protein with a central high mobility group box.[5,6] Referred to as the testis determining factor (TDF), this protein is responsible for differentiation of the testes, which secrete anti-Müllerian hormone (AMH) from Sertoli cells and testosterone from Leydig cells. Together, these hormones stimulate masculinization of the fetus. If the SRY gene is absent, the gonad differentiates into an ovary. Other genes play a role in sexual differentiation, such as Sox9,[3,7] an autosomal gene that aids in testis formation and is potentially upregulated by SRY; steroidogenic factor 1, a transcription factor which may also be regulated by SRY to increase levels of AMH;[8] and Wnt4, a growth factor involved in Müllerian duct regression and sex-specific cell migration.[9] In the bovine, expression of the SRY gene can be detected as

early as the four to eight cell stage embryo by reverse transcription polymerase chain reaction;[10] however, expression of the gene has not been strongly linked to the presence of the TDF protein, perhaps due to the inability to accurately measure TDF or the timing of protein production relative to SRY expression.[11]

History and various techniques of altering the sex ratio

Flow cytometry

Flow cytometry has many cell sorting applications and can be used to sort individual sperm cells by mass. Moruzzi suggested that differences in DNA mass could provide basis for separating X- and Y-bearing sperm.[12] Because of the X-chromosome's larger size relative to the Y-chromosome, X-bearing sperm have approximately 3.7-4.1% more DNA in their genome, depending upon species.[13] This subtle difference in DNA mass can be exploited by the flow cytometry process.

In order to distinguish between X and Y chromosomes, cellular DNA is stained with a fluorescent dye and a single stream of cells is forced through the flow cell and subjected to a light source. The fluorescence of each cell can be excited into scattering light at a lower frequency than the light source. The scattered light is detected and analyzed, allowing various parameters of the cell to be deduced, such as cellular mass, mass of DNA, proteins, pH, pigments, and enzymatic activity. As sperm cells pass through the flow cell, they can be selectively charged as positive or negative based on chromosomal content, and can then be deflected into separate paths as the sperm cells are attracted to either the positive or negative plate depending on which charge was applied. The current sperm sorting system permits the separation of six million X-bearing sperm and six million Y-bearing sperm per hour, at a purity of up to 90% depending upon species and differences among individual males.[2,14] After a semen sample is sorted via flow cytometry, the desired sperm cells are collected and used for artificial insemination or frozen for later use.

Although the accuracy of flow cytometry has been demonstrated using bull sperm, the process generally requires fresh semen and results in a reduction of sperm integrity. Another point of controversy is the bis-benzimidazole dye, Hoechst 33342, used to stain cells prior to flow cytometry. This stain targets DNA at adenine-thymine-rich regions of the minor groove and although chromosomal abnormalities have been suggested,[15,16] recent advances in the flow sorting technology have not only increased efficiencies and decreased costs,[17] but have also facilitated a very low incidence of genotoxicity *in vivo* in swine.[18] Depending upon semen quality, there may be bull to bull variation and sexed semen is only available from selected bulls, although the list of available bulls is growing sharply. The flow cytometer equipment costs approximately $250,000[19] and can only sort a limited number of cells per day; however, there are a growing number of sorting facilities available with several machines on site. The cost of a straw of sexed semen ranges from approximately thirty to fifty dollars and up to two-hundred dollars for dairy bulls, while the price is quite variable for beef bulls.[20] As efficiencies increase (currently around 90% accurate) and fertility approaches close to that of non-sexed semen, the costs are expected to continue to decrease to an economically feasible point for most dairy producers and a growing number of niche beef producers.[17] In addition, there may be variations in the flow cytometry accuracy process or resulting fertility among bulls of the same species or breed. Although the system is gaining momentum and there has been a substantial increase in the number of bulls from which sexed semen is available, the research applications and opportunities perhaps outweigh commercial utility.[14]

Surface proteins

Another suggested difference between X- and Y-bearing sperm is sperm head surface proteins. It is theorized that selectively binding proteins to X- or Y-bearing sperm will aid in their separation. The H-Y antigen has been studied to determine whether it is preferentially expressed on Y-bearing sperm and whether it could aid in sperm separation; results have been conflicting.[21, 22]

The H-Y antigen was first described by Eichwald and Silmser in 1955 following the observation that male-to-female skin grafts in mice were rejected while grafts within the same sex, as well as female-to-male grafts, usually succeeded.[23] In an attempt to exploit this difference in cell surface antigens, Sills

et al. treated human sperm with monoclonal immunoglobulin M (IgM) antibodies against the H-Y antigen and incubated the mixture with IgM antibodies appended to paramagnetic beads.[24] The preparation was sorted through exposure to a magnetic field, with anticipated results that the reactive group would be positive for the H-Y antigen and the non-reactive group would be negative. Fluorescent *in situ* hybridization (FISH) showed that 49% of Y- bearing sperm did not express the H-Y antigen; therefore, the usefulness of this approach has been limited.

Studies using mice have shown that developing X- and Y-bearing sperm express different genes; however, these products are shared among gametes due to the intercellular bridges formed during spermatogenesis.[25] Braun *et al.* used transgenic hemizygous mice with mouse Protamine 1 (mP1) transcriptional regulatory sequences fused to the human growth hormone (hGH) gene to demonstrate that RNA and protein can pass through the intercellular bridges among spermatids.[26] The hGH gene was transmitted to 50% of a developing sperm population, but subsequent immunocytochemical analysis showed that 90% of sperm contained hGH. The level of sharing may actually be greater; of the homozygous controls in the same study, only 92% were positive for hGH. Caldwell and Handel also demonstrated that post-meiotic spermatids share mP1 gene products through intercellular bridges.[27] Although sex-specific antigens have been identified on mammalian spermatozoa, exploiting this specificity has proven to be difficult and unrepeatable, therefore limiting the clinical application of this process.[25,28]

Albumin separation

Albumin separation is a technique that utilizes the progressive forward motility of sperm and possibly results in the isolation of Y-bearing sperm.[29] Semen is washed and diluted before being layered over columns of bovine serum albumin. Using this method, Ericsson *et al.* reported the isolation of 85% human Y-bearing sperm with up to 98% progressive forward motility.[29] Two years later, Ross *et al.* attempted to repeat the same experiment, but results failed to support Ericsson's findings.[30] Another study attempted albumin separation using bull semen with samples previously analyzed using flow cytometry. Results indicated that the ratios of X- and Y-bearing sperm were the same for the treatment group and the controls.[31] Ten years later, Ericcson co-authored a second study which found that 71% male offspring were produced out of 1,407 human births in 65 fertility clinics in the US and abroad through an albumin separation technique followed by intrauterine insemination on the presumed day of ovulation.[32] In contrast, an experiment with rabbits found that the sex ratio was unaltered by the separation of sperm through albumin gradient.[33]

It was previously believed that this separation process resulted in a sperm sample with a higher representation of Y-bearing sperm.[34] A group in Hong Kong determined through FISH that human sperm separation did not increase the concentration of Y-bearing sperm, rather, they postulated that serum albumin inactivated X-bearing sperm.[35] Because the mode of action of albumin separation is unknown and results are contradictory and unrepeatable, the utility of this approach has been limited.

Semen incubation

A study of *in vitro* produced bovine embryos showed that a 24 hour semen incubation period resulted in significantly more female hatched blastocysts when compared with zero or six hour semen incubation periods.[36] Watkins *et al.* found that human X-bearing sperm had significantly higher percentages of motility, rapid progression, faster curvilinear velocities, and hyperactivation after a 24 hour incubation when compared to Y-bearing sperm.[37] Because no differences in X- or Y-bearing sperm morphology or metabolism have been found, the effects of sperm cell incubation and associated mechanism(s) are unknown.

Commercially available products

Post-thaw semen treatment products named HeiferPlus™ and BullPlus™ (Emlab Genetics, Arcola, IL) are currently available which are claimed to "speed up" X- or Y-bearing sperm, respectively, to shift the sex ratio in favor of the desired sex. Due to the proprietary nature of the products, the

ingredients and mode of action are undisclosed. The manufacturer published internet resources supporting its claim in both hyperstimulated and naturally ovulating cattle, although no control groups were reported.[38] Recently, an independent trial investigated the effects of HeiferPlus™ treatment on semen used to breed both single ovulating cows and hyperstimulated cows compared to control groups. Results indicated that HeiferPlus™ did not effectively skew the sex ratio in favor of the female in either hyperstimulated or single-ovulating cows.[38] No independent research investigating the efficacy of BullPlus™ has been reported.

Timing of insemination

Another factor that may influence embryo sex is the timing of insemination relative to ovulation. Mammalian sperm can reach the oocyte quickly after insemination but may not be competent to fertilize the oocyte. They acquire competence as they move towards the ampulla of the oviduct, but will become unable to fertilize if they remain in the oviduct for an extended period of time.[40] It has been observed that uncapacitated sperm temporarily bind to the membranes of the oviducts in the isthmus and are released upon capacitation.[41] This event slows capacitation and lengthens the life span of the sperm, maximizing the probability of sperm being present in the ampulla to fertilize the egg even if insemination does not occur at a time coincident with ovulation.

OvSynch[42,43] is an ovulation synchronization protocol in which 100 µg gonadotropin releasing hormone (GnRH) is administered on Day 1, 25 mg prostaglandinF2α (PGF) is administered seven days later, followed by a second injection of 100 µg GnRH on Day 9. Pursley *et al.* synchronized ovulation with OvSynch and examined the timing of insemination relative to ovulation.[44] Cows were inseminated at 0, 8, 16, 24, or 32 hours after the second injection of GnRH. Cows inseminated at 0 and 32 hrs had the highest percentage of female offspring (61.0% and 65.0%, respectively), with the 0 hr group having the lowest pregnancy loss (between Day 35 and parturition) and the 32 hr group having the highest. This protocol has become widely used in cattle and more recently, sheep.[45,46]

Martinez *et al.* examined the timing of insemination relative to the onset of estrus in cattle and found that significantly more female calves (73.1%) resulted from inseminations performed from eight to 18 hours following first observed estrus.[47] A study in sheep revealed that more females resulted from inseminations five hours prior to ovulation and more males resulted from inseminations occurring five hours after ovulation.[48] Guerrero evaluated 1,318 human conception cycles from both natural conception and intrauterine insemination to determine if basal body temperature at insemination impacted the sex ratio of human births.[49] In natural conception, male births were most common (68.0%) when insemination occurred six days prior to the temperature shift, decreasing to 44.0% on the day of the shift. In artificial insemination, the trend was the opposite.[49]

In more precise human studies examining ovulation specifically, Wilcox *et al.*[50] showed that the timing of sexual intercourse relative to ovulation had no influence on the sex of the child, whereas Gray[51] reported that conception occurring close to ovulation resulted in a significantly lower proportion of male births.[50,51] Other studies have found no effects of the timing of insemination on sex ratio. Rorie *et al.* found no difference in sex ratio of calves resulting from inseminations either 20 hr or 10 hr prior to the expected time of ovulation.[52] In sows that had recently farrowed and weaned, Soede *et al.* demonstrated that the sex ratio was unaffected in sows inseminated at four hour intervals from the onset of estrus until ovulation.[53]

In a recent study, Angus and Angus-cross cows were subjected to ovarian hyperstimulation using follicle-stimulating hormone (FSH).[39] On Day 3 of FSH administration, two doses of 25 mg PGF were given 12 hours apart. Cattle were examined for signs of estrus every six hours and administered 100 µg of GnRH at estrus. Cattle were bred with either frozen-thawed semen that had been incubated for 20 minutes at 37 °C (control; n=12) or frozen-thawed semen that had been treated with HeiferPlus™[35] and incubated at 37 °C for 20 minutes, as per manufacturer's protocol (n=13). Chi square analysis revealed that the control group produced a significantly higher ($P<0.005$) proportion of female embryos (65/104, 65.0%) than the HeiferPlus™ group (71/167, 43.0%).

In two subsequent hyperstimulation studies with beef cattle, similar results were achieved.[54] Cows (n=8) and heifers (n=14), were synchronized, hyperstimulated, and examined for estrus 36 hours after the initial injection of PGF and every four hours thereafter until estrus was observed. Cattle were administered 100 μg GnRH at estrus and inseminated with two doses of frozen-thawed semen from a single ejaculate of one bull 12 hours later. The treatment groups for these studies were subjected to trans-rectal ultrasound every four hours from 36 to 76 hours after the initial injection of PGF while the controls were not subjected to ultrasound. Although there were no significant treatment effects, when grade 1-3 embryos (experiments 1 and 2; n=118) were combined the percentage of female embryos was higher ($P<0.05$) than the expected ratio of 50:50. This further supports the notion that synchronization of insemination and induced ovulation impacts the bovine embryo sex ratio.

Frequent rectal palpation/ultrasound

Ideta *et al.* found no difference in the sex ratio when hyperstimulated Holstein heifers were inseminated at either 48 or 56 hours after PGF with frozen-thawed semen from multiple bulls; however, in a second experiment, they reported that heifers which produced a high percentage of female embryos (>50%) had a shorter duration of estrus (13.2±3.9 hours vs. 24.9±6.5 hours), fewer standing mounts (27.8±5.9 vs. 75.0±18.5 mounts) and lower superovulatory response (8.4±1.0 vs. 19.7±3.1 ova collected) than heifers with a low percentage of female embryos (<50%), respectively.[55] Ideta *et al.* observed that heifers which had been subjected to ultrasonic evaluation of their reproductive tracts to examine ovulation patterns tended to produce a higher percentage of female embryos (66.7%).[56] These findings led to an additional study to determine the effects of frequent rectal palpation and ultrasonic evaluation on sex ratio.[57] Estrus synchronization was initiated by insertion of a progesterone-releasing pessary for nine days. A PGF analog was administered two days before the pessaries were removed and heifers were injected with equine chorionic gonadotrophin (500 IU, IM) concurrently with pessary removal. Hyperstimulation treatment began mid-cycle following estrus with eight decreasing doses of FSH. Prostaglandin was administered concurrently with the seventh and eighth FSH injections. The treatment group was rectally palpated and subjected to trans-rectal ultrasonography every four hours from 36 to 76 hours beginning with the initial PGF injection (concomitant with the seventh FSH injection) while controls were not subjected to ultrasound. All cows were artificially inseminated 56 and 72 hours after the initial injection of PGF with frozen-thawed semen from a single bull. Embryos were recovered on Day 7 after artificial insemination by means of uterine lavage. Sex of embryos was determined using loop-mediated isothermal amplification.[58] The percentage of female embryos (grades 1-3) was significantly ($P<0.05$) increased in the treatment group (67.8%) from the expected ratio of 50:50. The authors proposed that acute stress caused by frequent rectal palpation and ultrasound around the time of ovulation skewed the sex ratio towards the female.

In contrast, two studies by Davis *et al.* found that frequent ultrasound around time of ovulation did not significantly skew the sex ratio toward the female relative to the controls, which were not subjected to frequent ultrasound, although the controls had an unexpectedly high percentage of female embryos.[54] In Experiment 1, multiparous cattle were synchronized by ultrasound-guided follicular ablation, a progesterone-releasing pessary was inserted and cattle were administered 25 mg PGF. Superovulation was initiated 48 hours following follicular ablation with eight decreasing doses of FSH (once every 12 hours for four days). Pessaries were removed 48 hours after initiation of FSH and observations for estrus began 36 hours later and occurred every four hours for 40 hours. Gonadotropin-releasing hormone (100μg) was administered at observed estrus and cows were inseminated with two doses of frozen-thawed semen 12 hours later. Treated cattle (n=4) were subjected to trans-rectal ultrasound at each estrus detection period while controls (n=4) were not subjected to ultrasound but were processed through the chute. Embryos (control n=25; treatment n=23) were collected seven days after insemination, graded based on the International Embryo Transfer Society scale and sex was determined on grade 1-3 embryos (control, n=23; treatment, n=14) using duplex polymerase chain reaction (PCR)

and dot blotting.[59,60] The ratios of female to male embryos (grade 1-3) in the treatment and control groups were 57.1% (8/14) and 56.5% (13/23), respectively. The sex ratio did not differ significantly between groups or from the expected 50:50 ratio.

In Experiment 2, cattle were synchronized and hyperstimulated following a protocol similar to that used in Experiment 1. Multiparous cattle were synchronized by ultrasound-guided follicular ablation, a progesterone-releasing pessary was inserted and cattle were administered 25 mg PGF. Superovulation was initiated 48 hours after follicular ablation with eight decreasing doses of FSH (once every 12 hours for four days). Pessaries were removed 48 hours after initiation of FSH treatment and observations for estrus began 36 hours later and occurred every four hours for 40 hours. Additionally, since cattle were at random stages of the estrous cycle at the beginning of treatment, both groups were administered additional doses of PGF (25 mg) concomitant with the fifth and sixth FSH injection. The treatment group (n=8) was subjected to ultrasound every four hours in conjunction with the estrus detection periods. The control group (n=7) was neither subjected to ultrasound nor processed through the chute. Day 7 embryos (control, n=51; treatment, n=49) were collected and sex was determined on grade 1-3 embryos (control, n=41; treatment, n=40) using duplex PCR and dot blotting. The percentage of females for the treatment and control groups was 65.0% (26/ 40) and 73.2% (30/ 41), respectively. These data suggest that the timing of insemination relative to induced LH surge/ovulation (induced by GnRH) may have outweighed either the effects of ultrasound or the acute stressors (processing through the chute) associated with this experiment.

Other physiological factors

Oviductal proteins

Other studies have shown that an equal ratio of X- to Y-bearing sperm in a semen sample can result in a skewed sex ratio of offspring. Recently, oviductal proteins have been shown to interact and possibly influence gamete and embryo development. Oviduct-specific glycoproteins and osteopontin have been shown to affect sperm capacitation, gamete binding, fertilization, and embryo development.[61] It is possible that other secretions of the oviduct could influence the sex ratio through preferential binding of one sperm type or another to the oocyte or through a mechanism operating on or within the embryo prior to implantation. Catt *et al.* showed that of the total number of ovine embryos produced *in vivo*, a higher percentage of male embryos were represented, yet the sex ratio at birth was still 50:50 suggesting that more male embryos are lost perhaps during the peri-implantation period.[62] A study using *in vitro* produced bovine embryos found that culture systems using synthetic oviduct fluid medium with fetal calf serum resulted in significantly higher rates of male blastocyst survival when compared to other culture systems, although the mechanism is not understood.[63]

Cervical mucus

Martin found that sex ratio in human births was altered depending on the length of the follicular phase in women, with longer follicular phases resulting in more females.[64] The author suggested that properties of cervical mucus may differ depending on the length of the follicular phase, and that these differences may preferentially select one sperm type over another. In cows, Wehner *et al.* utilized an intravaginal probe to determine the conductivity of cervical mucus as a gauge for insemination times and their effect on sex ratio.[65] These authors reported that significantly more heifer calves resulted from inseminations that occurred when impedance values were low (35-45; ~20 hrs prior to expected ovulation) and significantly more bull calves resulted when impedance values were high (50-70; ~10 hrs prior to ovulation).

Follicular environment

At a local level within the follicle, an elevated concentration of testosterone can possibly alter fertilization events by producing an oocyte with an apparent preference for fertilization by a Y-bearing sperm cell. Recently, a higher proportion of *in vitro* produced male embryos resulted from oocytes

bathed in elevated follicular testosterone concentrations.[66] These data suggest that there are differences in the fertilization capabilities of oocytes related to the steroid environment, but not necessarily sex selection at the level of the sperm cell.

Drugs, environment, and occupation

Exposure to certain drugs and toxins has been shown to alter the sex ratio. Dibromochloropropane (DBCP), a toxic nematocide used extensively until the 1960's, has been shown to induce testicular dysfunction in workers subjected to high levels of exposure. Pregnancies conceived during exposure to DBCP resulted in 35% males whereas those same couples produced 53% males during the pre-exposure period.[67] Clomiphene citrate is a non-steroidal estrogen antagonist used to treat infertility by inhibiting estrogen binding by receptors in the anterior pituitary. This leads to increased production of FSH followed by LH and results in higher rates of ovulation. The use of clomiphene citrate also resulted in significantly fewer males at birth. Sampson *et al.* found that of 89 clomiphene citrate-induced ovulations followed by intrauterine insemination, 53.9% resulted in females.[68] Silverman *et al.* used clomiphene citrate in conjunction with albumin separation prior to intrauterine insemination and reported 51.9% females compared to the control group which had 48.6% females.[69]

Dioxin is a chemical produced as a byproduct of the manufacture of Agent Orange (2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid), an herbicide used extensively during the Vietnam War to defoliate plants and trees. Michalek *et al.* reported that dioxin exposure led to significantly more female offspring.[70] The Air Force Health Study examined offspring of veterans of Operation Ranch Hand, the unit that handled Agent Orange during the Vietnam War. Men who conceived children (n=181) less than one month post-service produced 56.9% females. After an industrial accident in Seveso, Italy, nine families in which both parents were exposed to dioxin resulted in 100 percent female children (n=12).[71] Data on the health of the resulting children were not reported. The National Toxicology Program classified dioxin as a human carcinogen in its Second Annual Report on Carcinogens (1981).[72] Davis *et al.* concluded that the reduction in male births should be viewed as a sentinel health event that could be linked to environmental factors such as pollution or dioxin exposure in industrialized countries.[73,74]

Retrospective studies have investigated the relationship between certain occupations and the sex ratio of offspring. Lyster examined the offspring sex ratio of Australian abalone divers.[75] Of the 58 participants in the study, there were 85 daughters (65.4%) and 45 sons (34.6%), all conceived after their fathers had become divers. Other studies have investigated the offspring sex ratio of pilots and astronauts exposed to high g forces. Pilots exposed to higher g forces had 60% female children, while those considered to work at low g forces showed no statistical difference in their offspring's sex ratio.[76] After administering a comprehensive questionnaire to 1,000 German pilots, Goerres and Gerbert of the German Air Force Institute of Aviation Medicine concluded that the offspring sex ratio of helicopter pilots significantly shifted in favor of females after the one-thousandth flying hour.[77]

Sex determination in non-mammalian species

Current methods of manipulating the sex ratio in mammals may not be applicable to other organisms. In avian species, the male is homogametic, designated ZZ, while the female is heterogametic, or ZW. In *Drosophila*, the Y-chromosome does not produce a male; a male is generated by the lack of two XXs. In other insects, such as bees, fertilized eggs become females while unfertilized eggs develop into males.[78] In most reptiles, sex is dependent upon the incubation temperature of the eggs, not by sex chromosomes deposited into the egg by a male. In species that display temperature-dependent sex determination, males and females have identical karyotypes.[79] Turtle eggs incubated at lower temperatures (26 °C) yield males, while those incubated at higher temperatures (33 °C) yield females. Alligators are the opposite, with lower temperatures producing females and increased temperatures resulting in males. Differentiation of gonads into ovaries or testes depends upon the incubation

temperature of the eggs during a period of development in which the embryo is thermosensitive. It is believed that temperature affects the undifferentiated gonads, since they are the site of estrogen synthesis and aromatase activity.[80]

Selecting for sex at the embryo level

Following semen sexing by flow cytometry, a single sperm cell of the desired sex can be injected directly into the oocyte *in vitro* through intracytoplasmic sperm injection. This technique assures an embryo of a chosen sex, but is invasive, inefficient, and can be damaging to the oocyte. *In vitro* fertilization (IVF) is a process in which the gametes are collected and fertilization takes place in culture. The resulting embryos can then be transferred into a recipient or frozen for later use. Studies describe a higher number of male offspring resulting from IVF, possibly due to the rapid early division and growth of male embryos relative to female embryos. Embryos at a more advanced stage in development are more likely to be selected for transfer rather than frozen.[63] Kochhar *et al.* theorized that because the Y-chromosome may contain more genes that function as transcription factors, development would be accelerated, whereas the X-chromosome contains genes that code for rate limiting steps required in embryonic developmental pathways.[81] Comprehensive understanding of the molecular events that control testes development, regulation of TDF, SRY, Sox9, and Wnt4 and to the extent to which these events control sex determination of subsequent offspring remain elusive.[3,82]

The sex of embryos can be determined prior to embryo transfer by PCR. This technique is useful because it requires only one or two blastomeres, leaving a viable embryo to be transferred into a recipient or frozen for later use. To determine the sex of a mammalian embryo, a DNA sequence specific to the Y-chromosome can be amplified and the product visualized using agarose gel electrophoresis. If a visible band is produced by the primer pair, the specific region of DNA is present and the embryo can be identified as a male. Lack of that band would signify lack of a Y-chromosome, indicating a female embryo. Polymerase chain reaction was first used for genetic diagnosis in bovine blastocysts in 1988 and is now a widely used method of sexing bovine embryos.[5, 83-87] Although it is sensitive and specific, PCR often yields inconsistent results when assessing samples with a limited template, especially when copies of target genes are unequal. Almodin *et al.* reported successful sex determination in 82.0% of embryos, using bovine embryos as models for human preimplantation genetic diagnosis.[88] A retrospective study conducted by Shea examined the use of PCR for sexing 4,183 embryos in a commercial bovine embryo transfer program.[89] Results showed that sex could successfully be determined in 90.0% of samples; failures at sexing were attributed to malfunctions of specific PCR reagents, contamination, and insufficient DNA template. Because PCR assays for embryo sexing have been optimized in order to amplify samples with small amounts of DNA, they are also sensitive enough to amplify contamination by a single DNA molecule.

Ethical considerations

While the purpose of altering the sex ratio in livestock is to reach financial goals, in human assisted reproduction, couples may want a child of a certain sex in order to avoid sex-linked genetic disorders, such as hemophilia, Duchenne muscular dystrophy, and fragile X syndrome, which are inherited through one of the sex chromosomes. Because males have only one copy of the X-chromosome, any gene present on the X- chromosome will be expressed, even if recessive. Reasons to desire a child of a certain sex may be for family balancing or to avoid sex-linked diseases; however, in India and China, there are strong politically-driven preferences towards males. A recent census in India showed that the male:female ratio of children under six years of age was 1000:927. In Haryana state, the ratio was smaller, with 1000:820.[90] In areas of China where the "one child" policy is mandated, the sex ratio is 117:100, but in rural areas of the country where multiple children are permitted, the sex ratio is balanced.[91,92] In most other countries, including the US, Canada, and the UK, census data show that no sex preference exists.[93]

Summary

Currently, there is no effective, reliable, inexpensive, and safe method for commercially swaying the sex ratio. Although flow cytometry is the most effective technique to date, it is expensive, may cause damage to sperm cells, and sexed semen is only available from a limited selection of bulls. A rapid increase in the flow-sorting technology efficiencies in the last few years has resulted in an accuracy >90% of the desired sex. Pregnancy rates using frozen, sexed sperm are within 70-90% of conventionally processed unsexed sperm, although the concentration of sperm cells per insemination is 10-20 fold less.[94] An extension of this technology has recently resulted in pregnancies from thawed sex-sorted semen which has been refrozen; however, as expected, pregnancy rates have been low and embryo wastage high.[95] To date no sperm cell surface proteins have been isolated that are specific to either X- or Y-bearing sperm and results of albumin separation studies have been conflicting. Although certain toxins and environmental factors appear to have an effect on the sex ratio, usually favoring females, the mechanisms are not well understood and the risks of exposure inarguably outweigh the benefits. Intracytoplasmic sperm injection, IVF, and embryo sexing require expensive equipment and supplies and are technically challenging, thereby limiting them mainly to human fertility treatments. Further, some aggressive techniques may have species-specific efficiencies.

Because it is difficult to exploit the only known difference between X- and Y-bearing sperm (mass), perhaps the sex of the embryo can be manipulated by the maternal side through oviductal or ovarian influences. A better understanding of the relationship of the timing of insemination relative to spontaneous ovulation, first observed estrus, or pharmacologically-induced ovulation in both hyperstimulated and single-ovulating cows warrants a more thorough investigation, as promising studies have been reported in cattle, sheep, and human studies.[45,47-49] In addition, these techniques could potentially be implemented at the farm level and without expensive equipment. Further research is necessary to determine and experimentally or clinically manipulate factors responsible for sex selection in livestock and humans.

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