Effect of sex on in vitro bovine embryo development

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ABSTRACT

In vitro fertilization has become a fairly standard practice for human infertility treatments and for the production of genetically superior dairy cattle. However, there is no consensus on the effect this method has on the sex ratio of the offspring. Some reports showed a tendency of higher male offspring production when embryo selection for transfer was performed at the blastocyst stage, while other studies failed to detect a difference in the offspring sex ratio. This study sought to determine whether there is a differential growth rate between embryos of different sexes and to determine if male embryos are more likely to reach the blastocyst stage than females. In vitro oocyte maturation, fertilization, and embryo culture were observed by time lapse video to determine the growth rates of male and female embryos. A fluorescent probe against the Y chromosome was used to determine sex. There was no difference in the growth rates of male and female embryos during any part of the developmental sequence to the hatched blastocyst stage ($p>0.05$). Based on these results, the culture conditions used in this study do not favor a selective growth advantage of one sex over the other. However, this study was limited by sample size ($n=15$ embryos analyzed under both time lapse and fluorescence microscopy), so further studies are needed to determine if these results hold true for larger sample sizes.

ABBREVIATIONS

Abbreviations are listed in alphabetical order of the abbreviation.

ART	Assisted reproductive technology
bp	Base pair
cAMP	Cyclic adenosine monophosphate
COCs	Cumulus-oocyte complexes
Cq	Quantification cycle
DAPI	4',6-diamidino-2-phenylindole
FCS (FBS)	Fetal calf serum (fetal bovine serum)
G6PD	Glucose-6-phosphate dehydrogenase
hpi	Hours post insemination
HPRT	Hypoxanthine phosphoribosyltransferase
IVC	In vitro culture
IVF	In vitro fertilization
IVP	In vitro produced
LRS	Lactated Ringer's Solution
mpf	Minutes post fertilization
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
OMM	Oocyte maturation media
PBS/PVP	Phosphate-buffered saline/polyvinylpyrrolidone
PCR	Polymerase chain reaction
PPP	Pentose phosphate pathway
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species

SE	Standard error
SOF	Synthetic oviductal fluid
SOF-BE1	Synthetic oviductal fluid - bovine embryo 1
SRY	Sex-determining region Y
TCM	Tissue culture medium
Xist	X-inactive specific transcript

INTRODUCTION

In vitro fertilization (IVF) is becoming an increasingly common procedure used to overcome infertility in humans and to improve reproductive efficiency in agricultural animals, such as through the production of more dairy cows from high genetic merit individuals. For dairy producers, female calves are desirable as they can be replacement animals for milking cows, and only a select few male calves of exceptional genetic merit are maintained as sires. Thus, ensuring that the embryo selection methods are not biased towards male offspring would have great economic benefits (22, 41, 42). For humans, control of offspring sex may be desired when a particular sex is at risk for genetic conditions, such as recessive X chromosome-linked diseases (51). Sex is the single characteristic that has the largest influence on mammalian phenotype and disease (29). Further, the ability to select for female dairy calves can improve overall farm efficiency, and this will indirectly benefit human society as well. Consequently, the ability to determine the sex of the offspring produced by IVF can be beneficial in multiple ways, so it is important to understand how our current methods are influencing the gender ratio of offspring in order to improve reproductive management techniques.

There is no consensus on the effects of IVF on offspring sex ratios nor the factors that may influence them (6, 20, 37). Some studies indicate that male embryos develop faster (46, 52), while other studies show that females do (20, 47). Further, some studies found no difference in sex-dependent growth rates (24, 32). The difference in development rates has been suggested to be the cause of the observed bias in human embryo transfer where male offspring are better

represented than females. This may be due to preferential growth and selection of male embryos for transfer (49). This was not observed when embryo selection was performed during the cleavage stage but was suggested to occur when embryos were selected at the blastocyst stage. Other results show that culture conditions have differential effects on the growth rates of male and female embryos of multiple species (4, 11, 46, 47). Several studies have shown that specific aspects of culture conditions affect embryo growth rates. For example, the presence of either glucose (26) or fetal calf serum (27) in the culture media inhibits embryo growth. However, whether male or female embryos are more susceptible to suboptimal conditions remains to be determined (18, 30). Therefore, understanding the different effects of in vitro culture conditions on early embryonic development of both males and females is important even for industries that otherwise do not seek to produce offspring of a given sex.

The selection of bovine embryos for IVF is performed at the blastocyst stage. If it is true that male embryos develop faster to the blastocyst stage in vitro, our culture methods may be selecting for the wrong gender. Therefore, the goal of this study was to determine if the embryonic growth rate is different between male and female embryos and if male embryos are more likely to reach the blastocyst stage than female embryos.

REVIEW OF LITERATURE

I. General Background

The ability to mature oocytes, fertilize mature oocytes, and culture early embryos in vitro has allowed the use of assisted reproductive technology (ART) to overcome infertility in humans as well as enhance the selection and production of genetically superior animals for agriculture. As the use of in vitro fertilization (IVF) for animal, human, and research purposes increases, it is imperative to understand the differential effects in vitro culture may have on male and female embryos (32). Although IVF is fairly successful at producing viable offspring, there are still many challenges associated with the use of IVF.

Currently, embryos are selected for transfer based primarily on morphological appearance (7, 9, 20, 29, 35, 44, 53, 54). In addition, the developmental stage of the embryo is used to determine which embryos are superior [*i.e.* which embryos are healthiest] (35, 44, 54). The developmental stage of the embryo is assessed alongside the time from fertilization in order to determine embryo development rates, which are also used to decide whether an embryo is transferred. Typical embryo growth analysis occurs at three major developmental stages: just after fertilization, at the cleavage stage, and at the blastocyst stage (20). Embryo growth is assessed in vitro because it is commonly believed that the fastest developing embryos are likely to be the most similar to in vivo produced embryos (23, 29, 36, 39, 57), and blastocysts that develop earlier have a greater chance of producing live offspring after transfer to a recipient uterus (9, 23, 29, 35, 36, 39, 44, 49). Embryos that cleave earlier are more likely to develop to the 8-cell stage

and are more likely to contain a greater number of cells at the time of transfer than embryos that cleave later. The early-cleaving embryos are also more likely to be chromosomally normal than the other embryos (29, 44, 57) and have a lower apoptotic index when measured at the blastocyst stage (29, 44). It is generally thought that the embryos that develop in a "timely manner" are those that are most likely to be developmentally competent and the most viable after transfer to a recipient uterus (29). However, the criteria for judging embryo quality were originally developed for assessing in vivo produced (IVP) embryos flushed and collected from donor females (53), which suggests they may not suit the assessment of embryos produced in vitro. Additionally, the amount of time an embryo takes to achieve the first cleavage division has a major impact on whether that embryo will develop to the blastocyst stage (36). The embryos with a significantly higher developmental capacity than the rest display the following cleavage pattern: at least two cells by 30 hours post insemination (hpi), at least eight cells by 48 hpi, and at least 16 cells by 72 hpi (29, 53). Although using the embryo growth rate to select superior quality embryos for transfer seems ideal because it is a quick, simple, and non-invasive technique, it is important to recognize that only approximately 10% of embryos display the aforementioned growth pattern (29), and the rate of development does not reveal everything necessary for judging an embryo's quality and viability, so only a few embryos would be available for transfer.

However, studies have shown that blastocyst morphology is an imperfect indicator of embryo viability or quality because the quality of IVP embryos is highly variable in general (20, 38, 39, 53, 54). For example, morphologically superior embryos can have severe chromosomal abnormalities (20). There is an additional challenge associated with using embryo morphology to

assess embryo quality. It is known that blastocysts frequently undergo repeated shrinkages and expansions to stretch the zona pellucida and eventually hatch from it. This causes their appearance to change dramatically, which increases the likelihood that the blastocyst will be incorrectly categorized [*e.g.* small versus large] (7, 24). The timing of blastocyst formation is the most important with regard to embryonic differentiation, though, and consequently, it may be a superior diagnostic measure to use in selecting embryos that will most likely result in pregnancy after transfer (53). It should be recognized that early-cleaving embryos are not always of superior quality (20, 29), and consequently they are not always the best embryos to transfer into a recipient uterus. Additionally, there is not a morphological grading system currently available that can accurately predict embryo sex (35).

It should be noted that even though embryo grading based on morphology is imperfect, it has some correlation with embryo developmental potential and chromosomal normality (20, 31, 53). For example, morulae graded at lower levels [*e.g.* as "poor" or "fair"] form significantly fewer blastocysts and have decreased hatching abilities than embryos graded at higher levels [*e.g.* as "good" or "excellent"]. Furthermore, hatched blastocysts graded at higher levels have more cells in total than those graded "fair" and "poor". Blastocysts developed from morulae graded as "excellent" have significantly more cells in the inner cell mass than blastocysts developed from "poor" morulae (53). Additionally, embryos at higher grades result in greater pregnancy rates than embryos at lower grades (31). Finally, even though embryos with excellent morphology still have high incidences of chromosomal abnormalities, embryos with poor morphologies have even

greater likelihoods of having chromosomal abnormalities (20). It is important to maintain the distinction between embryo developmental potential and embryo quality though (53).

Further, it is imperative to distinguish between slow-developing embryos that are not developmentally competent and slow-developing embryos that are developmentally normal but just grow slower (29). The slow-developing embryos should not be excluded from transfer simply because they are slow to develop. In some cases, these embryos may be of higher quality and viability than fast-developing embryos.

II. Effect of Sex on Embryonic Growth Rates

A. Differential Growth Pre-Transfer: Early Embryonic Growth In Vitro

1. Preferential Male Development

Results from numerous studies indicate there may be an effect of embryo sex on embryo development rates (47). Indeed, the differences in birth weights between male and female offspring have been attributed to different male and female prenatal growth rates (33). Several studies have shown there to be a faster growth rate of male embryos than female embryos in vitro (3, 4, 5, 6, 9, 12, 13, 14, 18, 25, 26, 29, 30, 33, 34, 35, 36, 37, 39, 40, 41, 43, 44, 46, 47, 50, 52, 54, 56, 57, 58, 59). The results of these studies indicate that male embryos accumulate a greater number of cells than female embryos within a given period of time, allowing male embryos to progress through some developmental stages more quickly than female embryos (14, 18, 26, 30, 33, 35, 52, 54, 57). Additionally, more male bovine embryos cleave to the 2-cell stage within the first 30 hours post insemination (hpi) than female embryos, suggesting the first

cleavage division occurs earlier in males than females (33). Male embryos not develop more quickly but also develop to more advanced stages in culture than female embryos (18, 25, 26, 27, 34, 57), becoming blastocysts, fully expanding, and hatching sooner than females (9, 13, 21, 29, 30). Other studies have shown that female embryos are more likely to stop development at the morula stage than males (25). Of the fast-developing embryos, a significantly higher percentage are male (about 80%) (3, 6, 13, 14, 40, 52, 54, 57). Of the slow-developing embryos, a significantly lower percentage are male (about 40%) (3, 6, 13, 14, 52, 54, 57). However, it is interesting to note that early during development in vitro, the sex ratio does not differ from the expected 1:1 (13, 47). Studies have shown that male embryos have a lower average mitotic index than female embryos (18, 56). Although this initially appears contradictory to evidence suggesting faster male embryo growth rates, this in fact is further support for the claim because male embryos reach more advanced developmental stages in vitro, causing them to reach their mitotic index peak and then display a decrease in the mitotic index. Female embryos do not reach the same developmentally advanced stages in vitro due to their average slower growth. Hence, female embryos tend to have a higher average mitotic index than male embryos (18, 56). The faster development of male embryos can skew the sex ratio of embryos selected for transfer to recipients, and this would ultimately impact the sex ratio of the offspring (12, 13, 35, 59). Indeed, preferential selection of male embryos for transfer has been suggested to be responsible for excess male births (2, 9, 12, 29, 35, 49, 59).

2. Preferential Female Development

Others have suggested the opposite sex-dependent developmental rates, proposing that female embryos cleave significantly faster than male embryos. Although the sex ratio in one study was equal during development, significantly more females were found to be early-cleaving embryos than males (20). In another study, female embryos tended to form blastocoeles earlier than males, indicating that females develop more quickly. However, these results were not statistically significant (47). The results of these studies directly oppose results indicating faster male development. Although multiple studies suggest that female embryos develop faster in vitro than male embryos, substantially more published papers claim the opposite.

B. Differential Growth Post-Transfer: In Vitro Produced Embryo Growth In Utero

The sex ratio of offspring born from IVP embryos is skewed with substantially more males than females (27, 33), which is not beneficial for dairy producers. The reason for this skewed gender ratio has been hypothesized to be due to the faster developmental rate of male embryos in vitro because blastocysts at more advanced developmental stages are selected for transfer (33).

However, the results of other studies do not support the conclusion that sex ratios greater than 1:1 at birth are caused by preferential male embryo selection for transfer. Rather, they posit that the greater number of male offspring at birth is due to the increased mortality of female embryos early after implantation, which may be caused by abnormal X chromosome inactivation (2, 50).

C. Distribution of X and Y Chromosome-Bearing Sperm

It is important to note that in all cases, the ratio of X-bearing to Y-bearing sperm is 1:1 (50). This has been confirmed through the use of the zona-free hamster assay (32). Therefore, any observed deviations from a 1:1 sex ratio in vitro are not due to the presence of a greater proportion of one type of sperm. This 1:1 sperm ratio holds true even after a variety of methods are used to prepare sperm for IVF. Furthermore, the preparation techniques do not alter the time it takes for either X- or Y-bearing sperm to fertilize oocytes, nor do they alter the time it takes for the resulting embryos to develop (24). Additionally, there is no evidence to support a different fertilizing ability of X-bearing versus Y-bearing sperm. Therefore, the likelihood of an oocyte being fertilized by a sperm cell carrying either sex chromosome is the same, and the expected sex ratio of the offspring is 1:1 (28).

III. Reason for Sex Effect 1: X and Y Chromosomes

Of the studies proposing a difference in embryo growth rates based on sex, multiple explanations for this observation have been proposed. Differences between the sex chromosome dosages in male and female embryos can affect embryonic development (10, 29, 30, 47). For example, some of the genes encoded on the Y chromosome impact male growth rates, allowing male embryos to develop faster than female embryos (11, 20, 30, 57). Some of those genes act as transcription factors [*e.g.* zinc finger protein Y (ZFY) and sex-determining region Y gene (SRY), both of which are known to have mitogenic properties], thereby accelerating development (25, 30, 33). However, sex-related differences occur in the absence of the SRY gene, so other factors affect sex-related embryo growth as well (25).

Additionally, some of the genes encoded on the X chromosome serve as rate-limiting steps in embryo metabolism pathways as well as in stress reduction pathways. X-inactive specific transcript (Xist), which is responsible for initiating the inactivation of the second X chromosome, is first detected at the blastocyst stage in cattle. Inactivation of the second X chromosome is not complete until several days after the process begins, leaving XX embryos susceptible to the effects of double X chromosome-linked gene expression. This amplifies sex-related growth differences as the maintenance of two active X chromosomes is known to slow mouse embryo development, and the same likely holds true in cattle (11, 17, 20, 21, 25, 26, 30, 33, 43). The period in which embryos display a difference in sex chromosome composition due to the double X chromosome-linked gene expression in female embryos is the period in which embryos are most susceptible to sex-dependent embryonic death (29). Further, the genes responsible for the control of the amount of oxygen radicals as well as those responsible for glucose uptake and metabolism [*i.e.* hypoxanthine phosphoribosyltransferase (HPRT) and glucose-6-phosphate dehydrogenase (G6PD)] are encoded by the X chromosome. The expression of these genes may result in female embryo developmental delays (11, 25, 26, 30, 43, 49, 59). Further support for early male blastocyst formation comes from the lower level of oxygen radicals in females (25). This is because oxygen radicals stimulate intracellular calcium release and increase proto-oncogene expression (46). Additionally, alpha-galactosidase, which assists in the metabolism of complex sugars and fats, and phosphoglycerate kinase, an enzyme involved in glycolysis, are expressed concordantly with HPRT (48).

Nuclear proteins encoded by the sex chromosomes may also play a role in the regulation of transcription (56). This would seem to have an impact on embryo growth as sex-chromosome-linked gene expression can be detected as early as the 2-cell stage (17, 33). In fact, results have shown that the difference in gene expression between male and female embryos can cause differences in energy metabolism, environmental sensitivity, and developmental kinetics (21). These effects were also demonstrated in studies performed in mice. Fetuses with a single sex chromosome (XO) were the same size as XY littermates and larger than XX littermates. This supports the role of the X chromosome gene expression level in regulating early embryonic growth (33). Other studies have shown similar sex-dependent growth rate differences, noting that IVP male embryos grow faster in culture than females during the first seven to eight days of culture, which is the time period soon after activation of the embryonic genome (11, 13, 25, 27, 40, 43, 52, 56, 57).

IV. Reason for Sex Effect 2: In Vitro Culture Conditions

In some species, sex-dependent embryo growth has been observed in vitro but not in vivo. This suggests that the in vitro culture conditions themselves may affect embryo growth (4, 11, 24, 25, 26, 27, 29, 33, 34, 38, 52, 53, 59). For example, it has been shown that male embryos develop faster than female embryos in Ménézo's B2 medium, but there is no difference in the growth rate of male and female embryos in tissue culture medium (TCM)-199. Further, all embryos are negatively impacted when cultured in TCM-199. Both males and females are slower to develop in TCM-199 than in Ménézo's B2 medium (31). Part of the difference between these media is the energy substrate they contain, which implies that the type of energy available in the medium

may impact development in a sex-dependent manner (10, 33, 43, 59). Indeed, studies have shown that male embryos develop faster than female embryos in the presence of high glucose concentrations (11, 24, 25, 30, 33, 36, 38, 47). This is partially due to differences in male and female embryo metabolic rates (13, 18, 21, 25, 26, 30, 36, 47, 59) as males are able to metabolize glucose faster than females (10, 24, 30, 46, 57). The growth-stimulating effect of oxygen radicals may also be a reason for the faster growth of male embryos in the presence of glucose (26, 46) since the glucose concentration in the culture medium is associated with the production of reactive oxygen species (ROS) (43).

Although oxygen radicals may be important in regulating growth, they are also known to be severely detrimental to embryo growth and development. The abnormal accumulation of intracellular glycogen is associated with a developmental block. Hydration of this glycogen store causes blastomere swelling, which disrupts multiple cellular processes and sometimes leads to cell lysis (46).

One of the proposed reasons for the difference in male and female embryo glucose metabolisms is because G6PD, which is the enzyme responsible for catalyzing the first (and rate-limiting) step of the pentose phosphate pathway (PPP), is encoded on the X chromosome. The PPP plays an important role in embryo metabolism as it is responsible for producing nicotinamide adenine dinucleotide phosphate ($NADP^+$), which is used in the synthesis of many complex molecules, such as lipids and ribose-5-phosphate, the precursor of all nucleotides (43). NADPH (reduced $NADP^+$) plays a critical reducing role in several important processes, including oxidative stress

defense. The oxidation of G6PD to ribose-5-phosphate and carbon dioxide during the PPP is an important source of NADPH. In order to respond to oxidative stress, embryonic cells can increase the rate and intensity of the PPP (30). G6PD is initially expressed at higher levels in female embryos. In agreement with this, results have shown that the PPP activity is four-fold higher in female embryos (10, 25, 30, 43). However, total glucose metabolism is approximately two-fold higher in male embryos (10, 24, 30, 57).

Glucose acts to partially inhibit HPRT activity. HPRT is important in purine salvage, so an inhibition of HPRT decreases the production of genetic material, an important process in embryo development. Furthermore, the same molecules responsible for inhibiting HPRT activity are able to induce the 2-cell block during development. Additionally, HPRT inhibition can cause the production of hydrogen peroxide, which stimulates further glucose accumulation, thereby substantially magnifying the effects of even a small amount of glucose in the culture medium (46). Since HPRT activity is linked to the X chromosome, and female embryos have twice the dosage of X chromosome-linked genes, this supports the observations of accelerated male embryo development in the presence of glucose and further skewing of the sex ratio towards males as the glucose concentration increases.

In fact, study results have shown that male blastocysts have substantially higher numbers of genes responsible for metabolic, mitochondrial, and cell cycle processes than female blastocysts. In the same study, it was found that female blastocysts have greater numbers of genes involved

in enzyme inhibition than male blastocysts (29). These metabolic differences may occur as early as the first cleavage division in bovine embryos (36).

Yet the results of other studies show that the effects of the presence of glucose in the culture medium are not so straightforward. Embryos were cultured in media containing glucose, and the sex ratio was measured on multiple days throughout culture. On Days 7 and 8 of culture, the presence of glucose did not affect the embryo sex ratio. In either the presence or absence of glucose, a significantly higher proportion of males than females was found in the group of expanded blastocysts. Although there was a deviation of the sex ratio from the expected 1:1 on Days 9 and 10 of culture, this only occurred when glucose was not present in the culture medium. A significantly larger proportion of the embryos that only developed to morulae by Day 10 was female. These results suggest that when glucose is included in the culture medium, there is a greater loss of female embryos (26).

High concentrations of glucose have been shown to affect embryos of both sexes. For example, in one study, high glucose concentrations were found to decrease both total cell numbers and cell numbers of the trophectoderm. The number of cells in the inner cell mass was not affected. This was observed in both male and female embryos. Additionally, glucose has been shown to reduce the number of viable blastocysts that develop. Again, these effects are not sex-dependent (10, 26, 46). However, embryos require glucose for post-genome activation development, so there must be a minimum amount of glucose in the culture medium for the embryos to survive and develop.

This is because glucose is a significant source of cellular energy, which helps ensure the rapid proliferation of early embryonic cells (46).

Additionally, the presence of fetal calf serum (FCS) in synthetic oviductal fluid (SOF), the medium used for culture of embryos, enhances male embryo survival in part by causing faster development and increased blastocyst production (27, 38, 47). Further, male embryos are able to reach the blastocyst stage in a shorter amount of time than female embryos when they are cultured in SOF supplemented with FCS (27). FCS also causes a higher level of apoptotic cells in female versus male embryos. Higher levels of caspase 3, which is responsible for the initiation of apoptotic DNA fragmentation through inactivating inhibitors of DNase and activating endonucleases, were found in female embryos and confirmed the observation of greater apoptotic rates in female embryos (21). Together, these components alter the sex ratio from the expected 1:1 male:female ratio. Without the presence of FCS, there is no effect on the blastocyst sex ratio, resulting in the production of equal male and female embryos (25, 27).

V. Sex Dependent Impact of In Vitro Culture (IVC)

Male embryos are less impacted by inadequate culture conditions [*e.g.* suboptimal culture medium, oxygen concentration, use of somatic cell co-culture] than female embryos (14, 18, 25, 26, 32, 38, 50, 57). This is partially because male and female embryos may have different abilities to mount stress responses during early embryonic development (29, 30). This is reasonable if male embryos develop faster than female embryos since studies have shown that the faster-developing embryos have better cellular oxidative stress defenses (29). The

faster-developing embryos have greater expressions of an $NADP^+$-dependent isocitrate dehydrogenase, which modulates oxidative damage, and RAD50, which is involved in DNA double-strand break repair. Together, these two genes help protect embryos from the negative impacts of stress (29). For example, exposure to temperature fluctuations during culture and transfer results in a loss of significantly more female embryos than male (25, 33). Additionally, there is a greater loss of female embryos at early stages of development after manipulation [*e.g.* transfer between IVF wells] or long-term culture (18, 25, 27, 32). However, exposure to time-lapse environments results in a loss of more male than female embryos (33), and Carvalho *et al.* found that prolonged culture did not impact embryos in a sex-dependent manner (13), so the sex-dependent ability of embryos to respond to stressful environments has yet to be fully elucidated (30). Overall, there are sex-dependent impacts on cell number, developmental rate, metabolism, and survival in culture (29, 47, 50). Both male and female embryos are impacted by suboptimal culture conditions, including pH and temperature changes (7, 21, 26, 29, 30, 38, 45, 47). Furthermore, too many nutrients can be just as detrimental to embryo growth as insufficient nutrients, resulting in epigenetic changes, impaired embryo development and blastocyst hatching, embryo death, and/or long-term health consequences for the offspring (10). Therefore, it is important to control environmental factors [*e.g.* inorganic ions, buffers, gas composition, amino acids, growth factors, vitamins, and macromolecules] when culturing embryos (7, 13, 16, 46, 59). However, the embryo quality is generally higher for embryos that develop in vivo than for those that develop in vitro. In vitro culture can negatively impact "epigenetic gene regulation, including DNA methylation maintenance, histone modifications, and expression of epigenetic modifiers" (29).

VI. Preferential Female Loss

Of particular interest, Gutiérrez-Adán *et al.* found that there was not a difference from the expected 1:1 sex ratio in 1-cell zygotes 24 hpi nor in 2-cell embryos 40 hpi in culture despite there being a difference in blastocyst sex ratio in favor of male embryos. These results suggest that there is a preferential loss of females during IVC to the blastocyst stage (25).

VII. Preferential Loss of Males

Several other studies have shown that there tends to be a greater loss of male embryos during development in vitro. This is in part because male embryos are believed to be more fragile than female embryos. This was shown through an experiment assessing fertilization rates in suboptimal conditions. It was found that fewer males than females were conceived in suboptimal conditions (30). King *et al.* found that "among abortuses and stillborn calves, 60% were male" (32). Although Carvalho *et al.* found that early in development there is a higher percentage of male embryos that develop to more advanced stages, when assessing the overall sex ratio of the offspring, there was no difference from the expected 1:1 sex ratio (13). Therefore, it is likely that male embryos die earlier than females (30). Other studies support the suggestion of the preferential loss of males based on a high sex ratio early in development and a sex ratio at birth that is not different from the expected 1:1 ratio. It has been suggested that a greater male fetal mortality rate later in development is perhaps the reason that a greater male:female sex ratio earlier in gestation still generates the expected 1:1 sex ratio at birth (50).

VIII. Stress

If it is true that more female offspring are born under conditions of stress, the Trivers and Willard "sex allocation hypothesis" should hold true (51). This hypothesis essentially poses that natural selection would favor a male:female sex ratio greater than 1:1 only during good conditions. Otherwise, it is beneficial to produce more female offspring to increase the likelihood of the survival of the species since one male can breed substantially more times in a given breeding season than one female, thus allowing one male to contribute more to the gene pool of the offspring than one female. The data suggesting a loss of male embryos in suboptimal conditions supports this hypothesis.

IX. No Difference/Preferential Loss

King *et al.* did not observe a difference from the expected 1:1 sex ratio during the first week of development, and this does not support the suggested substantially greater loss of male embryos during early embryonic development (32). Regardless of whether it occurs, the offspring sex ratio cannot be used to determine the sex ratio earlier in development due to the possibility of a preferential loss of one sex (50).

X. Lack of Sex Effect

Still other studies have indicated that embryo sex does not impact embryo growth rate (10, 19, 24, 31, 32, 37, 47, 50, 55). They suggest that preimplantation male and female embryos exhibit similar morphologies, have the same developmental potentials, and do not cleave at different rates. Male embryos were not found to grow at a faster rate than female embryos. One study

pointed out that although the sex ratio was slightly male biased, it was approximately 50% and therefore was not deemed to be significantly different (2). These studies found no difference in the number of cells nor in the mitotic indices of male and female embryos (32, 55). Male and female embryos were found to cleave early at the same rate (55). Regardless of the embryo size [*i.e.* small, medium, or large], the sex ratio was not statistically different from the expected 1:1 sex ratio. Additionally, both XX and XY embryos were found to be equally likely to develop to the blastocyst stage and be chromosomally normal when blastocyst sex was controlled for (19, 55). That is, the embryos that develop to the blastocyst stage first exhibit a male:female sex ratio of 1:1 (24, 55). Results further indicated that whether cleavage-stage embryos or blastocysts were transferred, the offspring sex ratio was not affected and remained 1:1 (55). However, these studies have pointed out that culture in suboptimal conditions may have a sex-dependent effect, and in those conditions, one sex may develop faster than another (10). These studies also agree that embryonic developmental competency is correlated with early cleavage (55), but they posit that there is a 1:1 sex ratio of early-cleaving embryos, unlike other studies which found an effect of sex on embryo growth rates.

XI. Early Embryonic Development In Vivo

It is also important to note that studies have shown that in vivo, blastocyst production does not differ from the expected 1:1 sex ratio (27). Additionally, studies of calves have shown that the live offspring born have a sex ratio that is not statistically different from the expected 1:1 male:female ratio (32).

XII. Conclusion

Understanding the relationship between culture conditions, embryo development rates, and the ratio of male to female embryos is of particular importance in livestock production (25). Farmers often seek to manipulate the sex ratio of their animals in order to obtain more females to produce milk and offspring. Systematically altering the sex ratio of offspring could increase the efficiency of the animals on farms (22), helping to reduce environmental impacts and increase food production for the growing human population. This manipulation is of great economic importance, particularly in assisting farmers in improving their genetic progress (32). For example, lethal sex-linked recessive traits could be controlled (22, 51). Additionally, sex is the characteristic that has the greatest impact on mammalian phenotype and disease (29), so understanding the outcomes of IVF as they relate to sex are important for industries without inherent offspring sex preferences. As IVF becomes a more common practice in animal agriculture, identifying the conditions that lead to a deviation from the normal 1:1 sex ratio could have important commercial applications as well (13, 26, 42). Knowledge of the success rate [*i.e.* number of calves born, their viability, and neonatal and perinatal mortality] of IVF is important when considering its use and application in livestock production (41, 42).

The factors influencing embryo growth rates have been studied for many decades, but a consensus has yet to be reached on the optimal conditions for normal embryo growth [*i.e.* that prevent alterations in the embryo sex ratio, embryo genetics, and other markers of embryo development]. Thus, little is known for sure with regards to embryo development. There are multiple reasons for this. According to Beatty, "'Statistically significant' positive findings are

published but are offset to some extent by failures to confirm. Further, it can be assumed that all positive findings are published, but not all negative ones, thus introducing a bias into the world literature." (8) Additionally, the majority of the studies have failed to be appropriately selective when analyzing data (15, 22). Ultimately, it would be beneficial if the field could arrive at a consensus about the effects of culture systems on embryo development as using embryo morphology as a method of sexing would be relatively cheap, non-invasive, and easy. Further, this agreement would allow for the standardization of industry and research procedures, which would allow results to be compared directly The use of embryo morphology analysis to determine sex can also be used to determine the sex of embryos flushed from superovulated donors as well as IVP embryos (6, 42). Any studies in this field will help to further the scientific understanding.

MATERIALS & METHODS

The in vitro fertilization and culture process occurred without any delays between days [i.e. the embryos were not frozen and returned to culture at any point during the experiment]. The temperature and gas concentrations in the air were monitored during all incubations.

I. In Vitro Production of Embryos

First, embryos were produced through the use of in vitro fertilization (IVF) and in vitro culture (IVC). Without this step, there would not have been samples to analyze through time lapse video analysis and sex determination procedures.

A. Ovary Pick-Up and Transport - IVF Day -1

Bovine ovaries for oocyte collection via follicle aspiration were collected from the Cargill Meat Solutions slaughterhouse in Wyalusing, PA, about 1.5 hours from Cornell University. Ovaries were transported back to the laboratory in thermoses filled with warmed saline solution. Ovaries were then rinsed with warmed Lactated Ringer's Solution (LRS) once back in the lab.

B. Oocyte Collection - IVF Day -1

Light colored follicles less than 8mm in diameter were aspirated from the ovaries collected from Cargill Meat Solutions into 50mL conical tubes to allow for collection of the oocytes. After aspiration, oocytes were left to sit without agitation in the follicular fluid on a warming plate for

at least 30 minutes to prevent the loss of cyclic adenosine monophosphate (cAMP) as loss of cAMP could lead to lower blastocyst rates (1).

C. In Vitro Maturation (IVM) - IVF Day -1

The oocyte pellets at the bottom of the tubes were aspirated and added to 15mL conical tubes. Holding media (Supplemental Material 1) was added to the tubes until the final volume reached 9mL. The 15mL conical tubes were then inverted and poured into 10cm diameter Petri dishes. Good quality cumulus-oocyte complexes (COCs), defined as those with at least 3 layers of compact granulosa cells, homogeneously dark cytoplasm, and at least 100µm diameters, were selected and moved to 35mm Petri dishes with holding media. The selected COCs were then transferred to a secondary 35mm plate and washed several times before being matured in 5-well plates containing 400µL of oocyte maturation media (OMM) per 50 COCs (Supplemental Material 2). The COCs were incubated at 38.5°C with 5% CO_2 in air for at least 20-24 hours to complete the maturation process from the germinal vesicle stage to the metaphase II stage.

D. In Vitro Fertilization - IVF Day 0

Frozen semen straws from proven Holstein bulls [*e.g.* Travino (1H010686) and Lirr (1H02706)] (Genex Cooperative, Ithaca, NY) were thawed in water baths at 37°C for 30 seconds. Live sperm were seperated from dead sperm and cryoprotectant media by density gradient centrifugation. A double layer density gradient was produced by gently adding 2mL of 40% BoviPure (Nidacon International AB, Göthenborg, Sweden) on top of 2mL of 80% BoviPure in a 15mL conical tube without mixing. The semen was then deposited on top of the layered BoviPure solutions. The

tube was centrifuged at 400*g* and 37°C for 20 minutes. Dead sperm are lighter than live sperm due to the inability of the dead sperm to maintain osmotic homeostasis. Dead sperm also have densities between the 80% and 40% BoviPure, while live sperm have higher densities than the 80% BoviPure and form a pellet at the bottom of the tube. The supernatant was then removed, and the remaining pellet containing the live sperm was resuspended with 5mL BoviWash (Nidacon International AB, Göthenborg, Sweden). The tube was then centrifuged again at 400*g* and 37°C for 5 minutes to remove the BoviPure from sperm media. The supernatant was removed and the pellet resuspended in approximately 200µL of media that was left in the tube. The concentration of sperm in the original sample was calculated based on the number of sperm counted in the known volume multiplied by the dilution factor using a nucleocounter. The average sperm concentration was one million sperm per milliliter. The sperm-rich media was diluted 1:50 by adding 5µL of the sperm-rich media into a 1.5mL tube containing 250µL of S100 Detergent diluent (Chemometic, Allerød, Denmark). The detergent permeabilizes the sperm membrane to allow the propidium iodide fluorescent DNA stain to bind to the sperm head. A microfluidic chip with known volume was used to identify and count the number of sperm heads in the diluted sample. The sperm were then incubated at 38.5°C with 5% CO_2 in air for 15 minutes to allow capacitation to occur. The matured COCs in the metaphase II stage were then moved into 5-well plates containing 400µL of SOF-Fert fertilization media (Supplemental Material 4) where they were washed several times to be cleaned of debris. Sperm were added to the corner of each well of the 5-well fertilization plate containing COCs so the final sperm concentration in each well was 1 x 10^6 sperm/mL. The presence of live sperm was then confirmed using microscopy. The plate was incubated at 38.5°C with 5% CO_2 in air for 18-20

hours. The time of sperm addition in the fertilization plate was recorded to be later used as the fertilization time and date during the time lapse plate preparation.

E. In Vitro Culture - IVF Day 1

Putative zygotes were transferred by well to a 35mm dish containing hyaluronidase/holding media mixture and then immediately transferred again to a 1.5mL tube with as little media as possible. Each tube was then vortexed for 3.5-4.5 minutes to denude the zygotes. The contents of the tube were then transferred to a Petri dish containing holding media to dilute the hyaluronidase and allow retrieval of the denuded zygotes. The zygotes were washed in holding media and transferred to 5-well plates containing 400µL of SOF-BE1 embryo culture media (Supplemental Material 5) or directly into microwells for time lapse analysis. Once all the zygotes were transferred, the plate was incubated at 38.5°C with 5% CO_2 and 5% O_2 for 10 days.

II. Time Lapse Analysis

This step allowed for the semi-continual observation of embryo development, which allows for the identification of when embryos reach each developmental milestone in order to determine the embryo development rates.

Time lapse analysis was performed using the Primovision system placed in the low oxygen incubator set at 38.5°C with 5% CO_2 and 5% O_2. After denuding, putative zygotes were cultured in individual microwells in 8- or 16-microwell dishes. The system takes images of each microwell every 10 minutes for 10 days. Embryo development rates were determined using the

software provided, and the timing of cleavage to each developmental milestone [*i.e.* 2-cell, 4-cell, morula, blastocyst, and hatched blastocyst stages] were recorded for analysis. For the last 3 weeks of experiments, the protocol was changed where on Day 9 after in vitro fertilization, the time lapse plate was removed from the time lapse microscope but remained in culture in the incubator for an additional two days. This was done to allow another "new" time lapse plate to be analyzed during the early developmental stages every week as the previous culture period only allowed one replicate to be analyzed every two weeks. On Day 11 after in vitro fertilization, each embryo was saved individually in a labeled PCR tube and stored at -80°C for later analysis.

III. Cleavage Analysis - IVF Day 4

In an attempt to increase the number of blastocysts available for analysis (due to the low number of blastocysts in the time lapse plates), a second experiment was performed. The goal of this experiment was to determine if the ratios of male:female embryos are similar during the early embryonic development (which is analogous to the cleavage stage selection of embryos for IVF transfers) and at the standard blastocyst stage (which is analogous to the blastocyst stage selection of embryos for IVF transfers).

Putative zygotes were divided into two groups after fertilization. On Day 4 after in vitro fertilization, all cleaved embryos that were at least at the 4-cell stage were removed from Group 1 and placed into a different well on the same plate containing 400μL of embryo culture media for later developmental analysis. The purpose of this transfer was to determine if the sex ratio of the embryos that reached the developmental milestone [*i.e.* at least the 4-cell stage] at this time

point [*i.e.*Day 4] was different from the sex ratio of the embryos that had not reached the developmental milestone by this time point. The plate was then returned to the incubator at 38.5°C with 5% CO_2 and 5% O_2 for an additional four days.

IV. Blastocyst Analysis - IVF Day 8

The purpose of this step was to determine if the sex ratio of the embryos that reached the blastocyst stage at Day 8 was different from the sex ratio of the embryos that developed to the blastocyst stage after Day 8.

The second time point for the second experiment (part of the experiment in Section III) was Day 8 after in vitro fertilization [*i.e.* at the analogous time for embryo selection for transfer at the blastocyst stage]. All embryos that had developed to the blastocyst stage by Day 8 after in vitro fertilization were moved into a different well on the same plate containing 400µL of embryo culture media. The plate was returned to the incubator for an additional three days. All blastocysts were recovered and saved in individually labeled PCR tubes and stored at -20°C for later analysis.

V. Embryo Sex Determination by Quantitative Polymerase Chain Reaction (qPCR)

The purpose of this step was to attempt to determine the sex of the embryos through the use of qPCR. One set of primers (Supplemental Material 6 and 7) targeted the autosomal actin housekeeping gene. A second set of primers was designed to target the sex-determining region Y (SRY) gene on the Y chromosome, but this primer set was never tested due to issues that arose

during the optimization of the autosomal actin housekeeping gene qPCR trials and the time constraints of this study. Whole embryo genomes were individually amplified using the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO).

A. Single Cell Lysis and Fragmentation

The embryos were lysed and fragmented to allow the reagents to gain access to the embryonic genome. A Lysis and Fragmentation Buffer Solution Stock was prepared by adding 2µL of Proteinase K Solution into 32µL of 10X Single Cell Lysis and Fragmentation Buffer and vortexing the mixture. The embryo was thawed and re-suspended in a total volume of 9µL of water. Next, 1µL of the Lysis and Fragmentation Buffer Solution Stock was added to the embryo sample and mixed. The sample was then incubated at 50°C for 60 minutes and then heated to 99°C for four minutes. Finally, the sample was cooled on ice.

B. Library Preparation

Libraries of DNA from lysed embryos were prepared using the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO). The total sample volume of a lysed embryo (10µL) was transferred to a quantitative polymerase chain reaction (qPCR) 36-well plate. Next, 2µL of 1X Single Cell Library Preparation Buffer was added to each sample followed by 1µL of Library Stabilization Solution. The sample was then mixed and incubated at 95°C for two minutes. Then the sample was cooled on ice and centrifuged. Next, 1µL of Library Preparation Enzyme was added to the sample. The sample was then mixed and briefly centrifuged before being incubated at 16°C for 20 minutes, then 24°C for 20 minutes, then 37°C for 20 minutes,

then 75°C for five minutes, and finally held at 4°C. The sample was then centrifuged and either amplified immediately or stored at -20°C for up to three days before amplification.

C. Amplification

The entire embryonic genome was then amplified according to the directions provided in the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO) in order to increase the initial DNA sample size for use in the qPCR plate. The sample was thawed and 7.5µL of 10X Amplification Master Mix, 48.5µL of PCR quality water (Corning Inc., Corning, NY), and 5.0µL of Whole Genome Amplification DNA Polymerase were added. The sample was then mixed thoroughly, briefly centrifuged, and amplified in a thermocycler. Initial denaturation occurred at 95°C for three minutes and was followed by 25 cycles of denaturation at 94°C for 30 seconds followed by annealing/extending at 65°C for five minutes. The plate was then held at 4°C. After the cycling was completed, the sample was either used immediately in the qPCR process or stored at -20°C until use.

D. qPCR

The samples of amplified embryo DNA were diluted 1:10 and 1:20 with PCR quality water (Corning Inc., Corning, NY) in order to dilute any remaining chemicals from the prior amplification steps as they can be damaging to the embryonic DNA. The embryo samples were run in duplicate qPCR reactions. The adult male Holstein DNA controls were not amplified prior to qPCR and were diluted 5 times in a 1:5 serial dilution with PCR quality water. The MasterMix was prepared by adding 15.0µL of SYBR Green (Thermo Fisher Scientific, Waltham, MA),

7.2µL of PCR water (Corning Inc., Corning, NY), 0.9µL of Forward Primer (Supplemental Material 6), and 0.9µL of Reverse Primer (Supplemental Material 7) per well. The total primer concentration was 0.30µM per well. After preparation, the MasterMix solution was vortexed thoroughly and 24µL were pipetted into each well. Next, 6µL of each sample were pipetted into the appropriate wells. Four wells on the plate were filled with MasterMix and PCR water as a non-template control. The plate was then sealed and centrifuged for approximately 10 seconds. The qPCR plate was run at a range of temperatures consisting of 60.0°C, 60.3°C, 61.0°C, and 62.0°C (one temperature per row). The qPCR cycling conditions consisted of initial denaturation at 94°C followed by 34 cycles of denaturation at 96°C for 20 seconds, annealing at the assigned temperatures above for 20 seconds, and extension at 73°C for 10 seconds. The plate was then held at 4°C. After the cycling was completed, the plate was stored at -20°C until the products were examined on a gel.

Attempts were made to optimize qPCR for primers for the autosomal actin housekeeping gene to serve as a positive control of embryo genome amplification for the primers for the SRY gene. qPCR was never optimized using primers for the SRY gene due to failure of the autosomal actin housekeeping gene to accurately and reliably amplify in embryo DNA samples (*see Discussion*).

E. Post-qPCR Analysis

After the qPCR was complete, the results were analyzed in two ways. First, the melt curve derivative graph peaks were analyzed for uniformity and consistency. Ideally, the graph corresponding to each well would have only one peak that occurred in the same place as those of

the graphs for the other wells. Next, the average quantification cycle (Cq) was analyzed for each sample in order to determine the efficiency of the qPCR. To do this, the Cq values for each replicate of the same sample were averaged together and compared to the negative log of the sample concentration through a power analysis. This determined the percent efficiency of the run, an indication of how much product was produced per cycle. The R-squared value was also calculated to determine how closely the data points matched the trend.

F. Gel Electrophoresis of qPCR Products

A gel for electrophoresis was mixed with 1.200g of Agarose 3:1 High Resolution Blend (VWR International, Radnor, PA), 60mL of 1X TAE (Tris base, acetic acid, and EDTA) Buffer (Thermo Fisher Scientific, Waltham, MA), and 6µL of SYBR Safe (Invitrogen, Carlsbad, CA). The mixture was then heated in a microwave for five minutes. The gel was then poured into the mold and allowed to harden with 10-well combs. The gel was then loaded with 10µL of Quick-Load Purple 100 base pair (bp) DNA Ladder (New England BioLabs, Ipswich, MA), control adult male Holstein DNA, and experimental embryo DNA samples, and a non-template control. The DNA and non-template control were mixed with purple dye from the same ladder kit. The gel was run at 80V, 500mA, and 250W for approximately 30 minutes (until the bands were visible at approximately 3.5cm). The gel was then viewed under ultraviolet light.

VI. Embryo Sex Determination by Fluorescent Sex-Y Probe Staining

The purpose of this step was to attempt to determine the sex of the embryos through the use of a fluorescent probe due to the failure of the qPCR trials to determine the sex of the embryos. The embryos were sexed using a Y chromosome fluorescent probe from the Bovine Sex-Y Kit (MOFA Global, Verona, WI) with a modified protocol as outlined below.

Embryos were thawed and placed in a minimal drop of media on glass slides labeled with the embryo identifications. Embryos were crushed using thin, fire-pulled, glass pipettes to release individual cells. The media was encircled with a PAP pen (Sigma-Aldrich, St. Louis, MO) while it was still wet. The slides were allowed to air dry and then placed in a 60°C oven for one minute. The slides were then immediately transferred to a fixative comprised of a 15mL methanol:5mL acetic acid mixture deep enough to submerge the entire slide for five minutes and were then air dried completely. The embryos were then covered with 3µL of Sex-Y Buffer 1 for exactly five minutes and then immediately covered with 100µL of Sex-Y Buffer 2, drained, and re-covered with 100µL of Sex-Y Buffer 2 for one minute. The slides were drained by inversion and then air dried. Next, three tubes of the Sex-Y probe were resuspended with 500µL of Sex-Y Buffer 2 each. The embryos were each covered with 93.6µL of the Sex-Y probe and then placed in humidified slide chambers and warmed at 40°C for 35 minutes. The slides were then drained and washed with 200µL each of Buffer 2 for one minute. Next, the slides were each washed twice in 3µL of Sex-Y Biopsy Medium. The slides were drained and air dried and then covered with a 6µL drop of Sex-Y Mounting Medium. The slides were covered with glass cover slides. Finally, the embryos were located using the 10X objective lens under the 4',6-diamino-2-phenylindole (DAPI) filter (excitation 358nm; emission 461nm). The embryos

were then viewed using the 40X objective lens and the Rhodopsin filter. Digital images were taken for later analysis. Male embryos were identified as those with bright red dots within the cells. Female embryos lacked any bright dots and appeared generally red throughout the cell.

VII. Time Lapse Video Analysis

The purpose of this step was to identify when embryos reached each developmental milestone in order to calculate the embryo development rates.

The plates and individual wells the blastocysts were collected from were identified, and time lapse videos of each embryo were analyzed for important developmental milestones. The time the embryos reached the 2-cell, 4-cell, morula, blastocyst, and hatched blastocyst stages (if available) were recorded in terms of hours post insemination (hpi). The 2-cell stage was defined as when a clear division was visible between two cells. The 4-cell stage was defined as when four unique cells could be clearly visualized. The morula stage was defined as when the individual cells were no longer visible because they were too small to distinguish from each other (approximately 16 cells). The blastocyst stage was defined as the moment the blastocoel appeared. The hatched blastocyst stage was defined as when the blastocyst broke through the zona pellucida so the zona pellucida was no longer continuous.

VIII. Statistical Analysis

A Chi-Square Goodness-of-Fit test was performed using Minitab Express (Minitab Inc., State College, PA) to compare the number of male and female embryos observed with fluorescence microscopy to the number of expected male and female embryos [*i.e.* a 1:1 male:female ratio] . To determine if the embryonic development rates were different between male and female embryos, a Kaplan-Meier Survival Analysis was performed for each developmental milestone [*i.e.* 2-cell, 4-cell, morula, blastocysts, and hatched blastocyst stage]. This analysis determines the probability of male and female embryos reaching each developmental milestone within a given period of time. Each embryo was identified to be male or female based on the fluorescent probe result and the time to each milestone compared using JMP Pro version 12 (SAS Institute, Cary, NC).

RESULTS

I. Growth of Embryos In Vitro

Nine plates of 16 embryos each were used for this study. This is a total of 144 embryos. Only 19 total embryos developed to the blastocyst stage. This is a 13.2% blastocyst development rate. Out of those 19 embryos, only three hatched. This is a 15.8% hatching rate for the blastocysts and a 2.1% hatching rate overall. At least one blastocyst formed on each plate. One plate had two blastocysts form. Another plate had three blastocysts form. A third plate had four blastocysts form. It is important to note that fungus grew on one of the plates. On this plate, only one embryo developed to the blastocyst stage and then failed to hatch.

II. Quantitative Polymerase Chain Reaction (qPCR) to Identify Embryo Sex

The average quantification cycle (Cq) for each adult male Holstein control sample and the unknown embryo samples for the last [*i.e.* eighteenth] optimization are listed in Table 1. The average Cq values for the embryo samples were approximately the same and fell between the average Cq values for the 1:25 and 1:125 dilutions for the adult male Holstein control samples.

Table 1. Mean quantification cycle number (Cq) for adult male Holstein control samples and unknown embryo samples for the final optimization of qPCR.

Sample	Dilution	Cq Mean
Adult Male 1 Control	1:5	*27.15*
Adult Male 1 Control	1:25	*29.18*
Adult Male 1 Control	1:125	*31.48*
Adult Male 1 Control	1:625	*33.48*
Embryo Sample	1:10	*30.26*
Embryo Sample	1:20	*30.25*

Analysis of qPCR products obtained in the final optimization of the autosomal actin housekeeping gene by gel electrophoresis revealed different size products for the embryonic and adult DNA templates (Figure 1). The adult male Holstein controls (lanes 2 and 3) appeared at slightly less than 200 base pairs (bp). The embryo samples (lanes 4-9) appeared at approximately

300bp. The expected size of the products was 162bp. The melt curve derivative graph for this qPCR is shown in Figure 2. The first peak is the highest average temperature at which the change in fluorescence of the embryo samples was the greatest (approximately 80°C) and indicates the temperature at which the embryo sample DNA began to degrade. The second peak is the highest average temperature at which the change in fluorescence of the adult male Holstein control samples was the greatest (approximately 88°C) and indicates the temperature at which the adult sample DNA began to degrade. The difference in temperatures suggests that the products are of different sizes and indicates problems with the qPCR (*see Discussion*).

Due to the unexpected results and issues using the autosomal actin housekeeping gene during qPCR, the Y chromosome specific sex-determining region Y (SRY) primer set was never tested. Without consistent and correct amplification of the autosomal actin housekeeping gene, there would not have been a control for the qPCR trials, so false negatives during sexing would have gone unnoticed [*i.e.* embryos that did not amplify using the SRY primer set might have been inappropriately labeled female when in reality there might have been an issue with the embryo itself that caused it not to amplify using the SRY primer set].

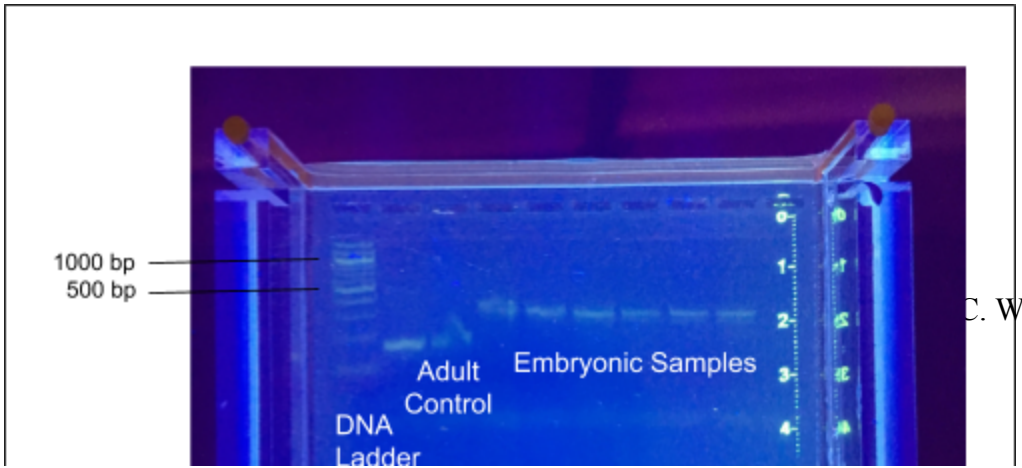


Figure 1. Gel electrophoresis of qPCR products obtained in the final qPCR optimization of the autosomal actin housekeeping gene. The 100bp ladder is in the farthest left lane. The adult male Holstein control samples are in the next two rightmost lanes and are just under 200bp. The unknown embryo samples are in the next six lanes and appear at about 300bp. The rightmost lane contains a sample from the non-template control showing the absence of a qPCR product as expected.

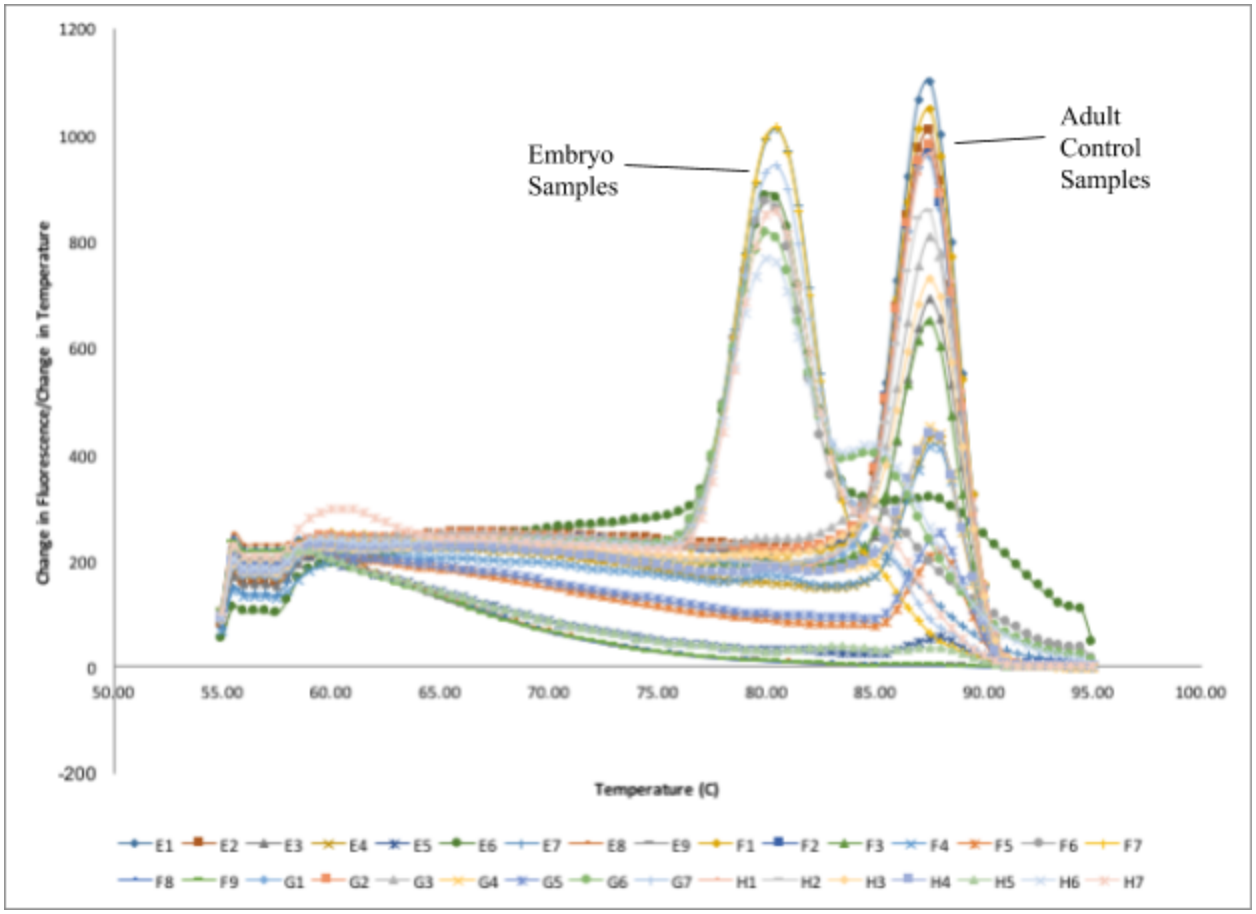


Figure 2. Graph of melt curve peaks from the final qPCR optimization. The x-axis represents temperature in °C, increasing from left to right. The y-axis represents the change in fluorescence divided by the change in temperature, increasing from bottom to top. The first peak is at approximately 80°C and corresponds to the time the embryo sample DNA began to degrade. The second peak is at approximately 88°C and corresponds to the time the adult male Holstein control sample DNA began to degrade.

III. Fluorescent Sex-Y Probe Staining to Identify Embryo Sex

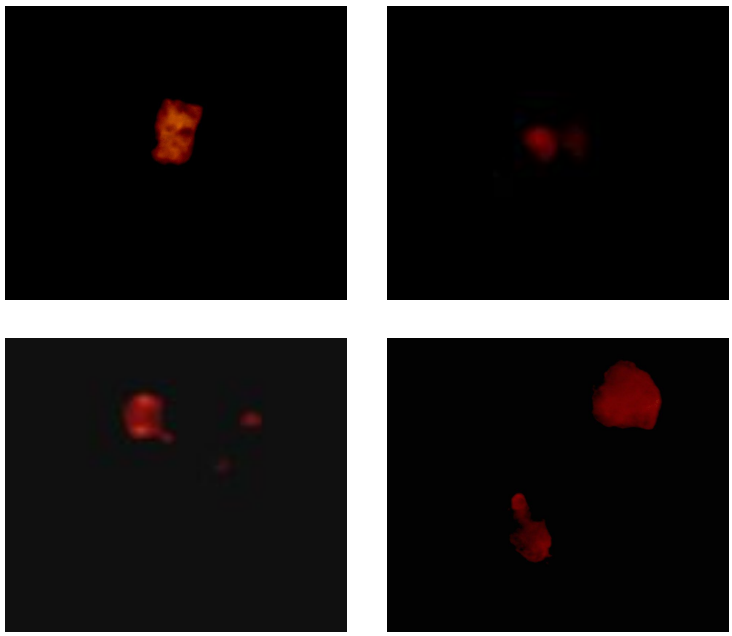
Out of the 19 blastocysts used for this study, only 16 were tested by staining with the Y chromosome-specific fluorescent probe. As per the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO), embryos that stained generally red or yellow with bright dots in the cells were male, and embryos that stained generally red or yellow and lacked bright dots were female. Nine of the stained embryos were female, and six were male. These numbers were not different ($p=0.4386$ as determined by the Chi-Square Goodness-of-Fit test). All embryos that underwent the staining process were able to be visualized under fluorescence microscopy. Images of representative male and female embryos can be seen in Figure 3. Embryo sex is also summarized in Table S1 (Supplemental Material 8).

IV. Time Lapse Video Analysis

Out of 15 total embryos analyzed, two embryos were unable to be accurately viewed during the first cleavage to form two cells and one embryo was unable to be accurately viewed during the

cleavages leading to the 4-cell stage. The amount of time it took each embryo to reach each developmental milestone is summarized in Table S1 (Supplemental Material 8). The average and standard error for the time for male embryos, female embryos, and all embryos to reach each milestone is summarized in Table 2 and Figure 4.

A. Representative Female Embryos:



B. Representative Male Embryos:

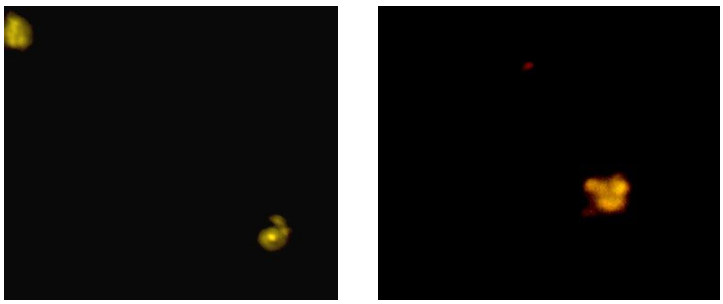


Figure 3. Representative images of embryos tested for the presence of the Y chromosome using a fluorescent Sex-Y probe. Female embryos shown in panel A appeared generally red or yellow and lacked bright dots in their cells. Male embryos shown in panel B appeared generally red or yellow with bright dots in their cells.

Note: some cells appear smaller than others as they were crushed more or folded during

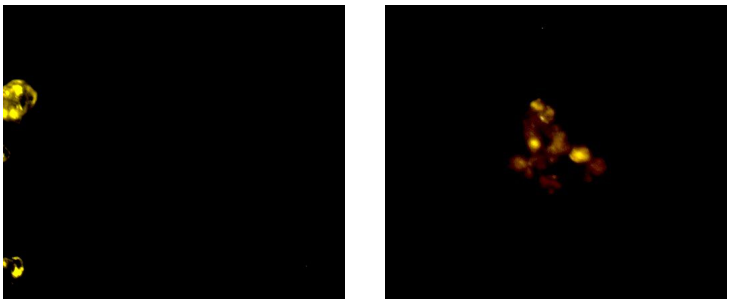


Table 2. Average time and standard error (SE) (in minutes post fertilization) for male and female embryos to reach developmental milestones: the 2-cell, 4-cell, morula, blastocyst, and hatched blastocyst stages. (n = number of embryos)

Stage	Male	Female	Total
	Average ± SE (n)	Average ± SE (n)	Average ± SE (n)
2-Cell	3,344 ± 205 (5)	3,349 ± 88 (8)	3,347 ± 96 (13)
4-Cell	5,041 ± 941 (6)	4,261 ± 165 (9)	4,595 ± 407 (15)
Morula	9,812 ± 523 (6)	9,630 ± 409 (9)	9,873 ± 334 (15)

Blastocyst	11,194 ± 519 (6)	11,458 ± 372 (9)	11,489 ± 312 (15)
Hatched Blastocyst	12,430 (1)	12,819 ± 291 (2)	12,689 ± 212 (3)

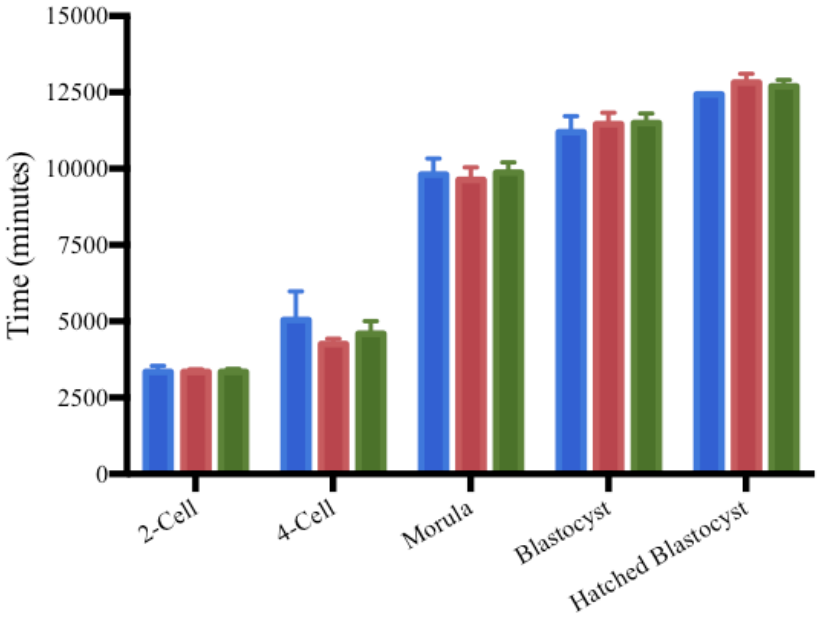


Figure 4. Average time and standard error (in minutes post fertilization) for embryos to reach developmental milestones: the 2-cell, 4-cell, morula, blastocyst, and hatched blastocyst stages. Male embryos are represented by the blue bars. Female embryos are represented by the red bars. All embryos combined are represented by the green bars.

Of the embryos that were observed to cleave to the 2-cell stage, the average time was 3,347 ± 96 minutes post fertilization (mpf) (average ± standard error) and the range was from 1,169 to 2,668 mpf. Of the embryos that could be observed reaching the 4-cell stage, the average time was 4,595 ± 407 mpf and the range was from 1,748 to 7,028 mpf. All embryos could be viewed during cleavage to the morula stage. The average time to reach the morula stage was 9,873 ± 334 mpf and the range was from 6,804 to 10,605 mpf. All embryos could be observed reaching the blastocyst stage. The average time to reach the blastocyst stage was 11,489 ± 312 mpf and the range was from 8,071 to 11,816 mpf. Only three of the blastocysts that formed became hatched blastocysts. The average time to the hatched blastocyst stage was 12,689 ± 212 mpf and the range was from 11,297 to 11,640 mpf. There was no difference between the proportion of male and female embryos that reached the blastocyst stage in this study ($p=0.4386$).

Results of the Kaplan-Meier Survival Analysis are shown in Figures 5-8. Two male embryos appear substantially slower during development to the 2-cell and 4-cell stages (Figures 5 and 6). These embryos took much longer than the other male embryos to reach those developmental

milestones. The male and female embryo development rates appear more similar and evenly distributed during development to the morula and blastocyst stages (Figures 7 and 8). No comparison was made between male and female developmental rates to the hatched blastocyst stage as only one male and two female embryos reached that developmental milestone.

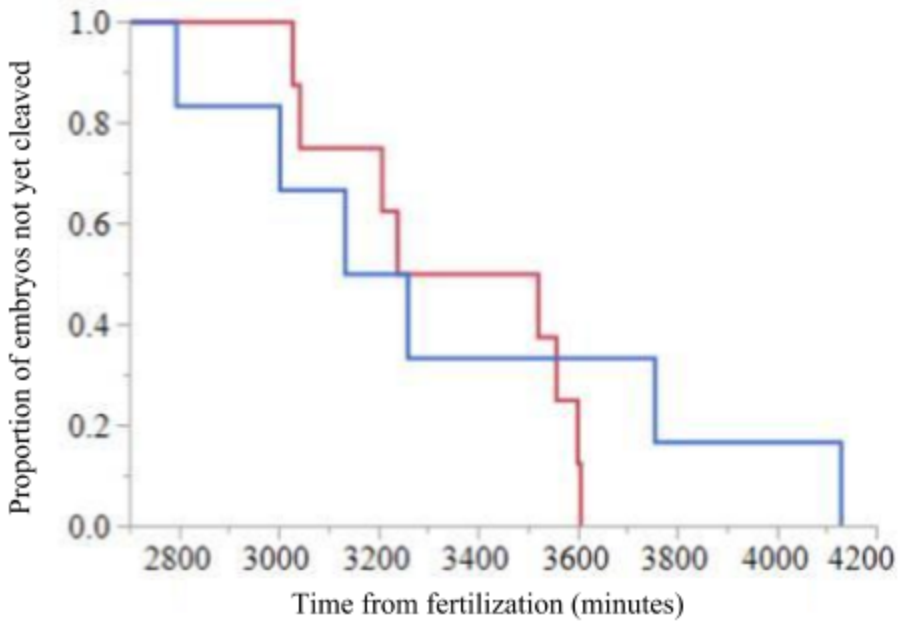


Figure 5. Kaplan-Meier Survival Analysis for development of male and female embryos to the 2-cell stage. Male embryos are represented by the blue line, and female embryos are represented

by the red line. The y-axis represents the proportion of embryos remaining at a given time point. The x-axis represents the time in minutes. Two male embryos appear substantially slower during development to this stage than the rest of the male embryos.

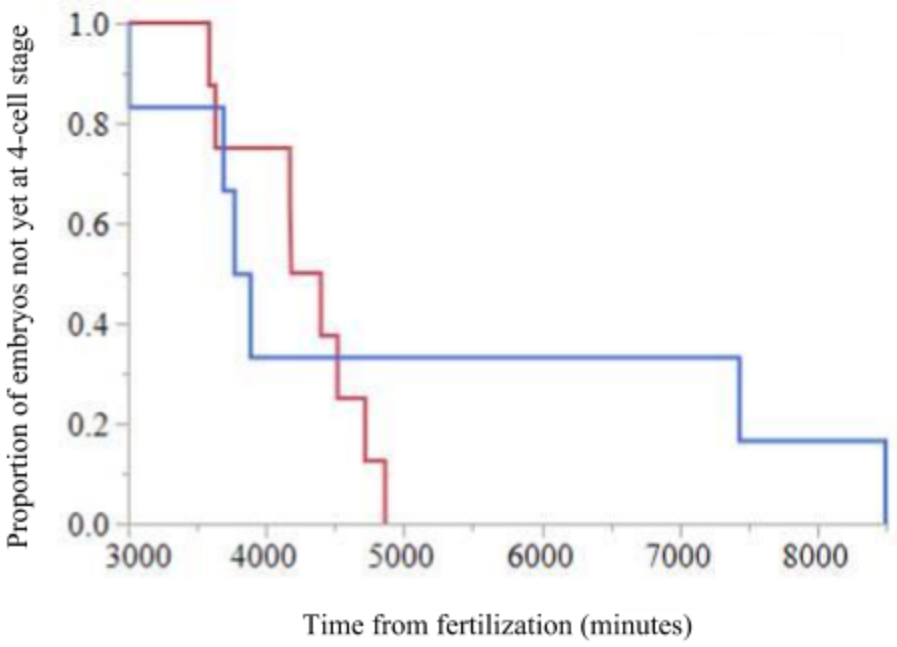


Figure 6. Kaplan-Meier Survival Analysis for development of male and female embryos to the 4-cell stage. Male embryos are represented by the blue line, and female embryos are represented

by the red line. The y-axis represents the proportion of embryos remaining at a given time point. The x-axis represents the time in minutes. Two male embryos appear substantially slower during development to this stage than the rest of the male embryos.

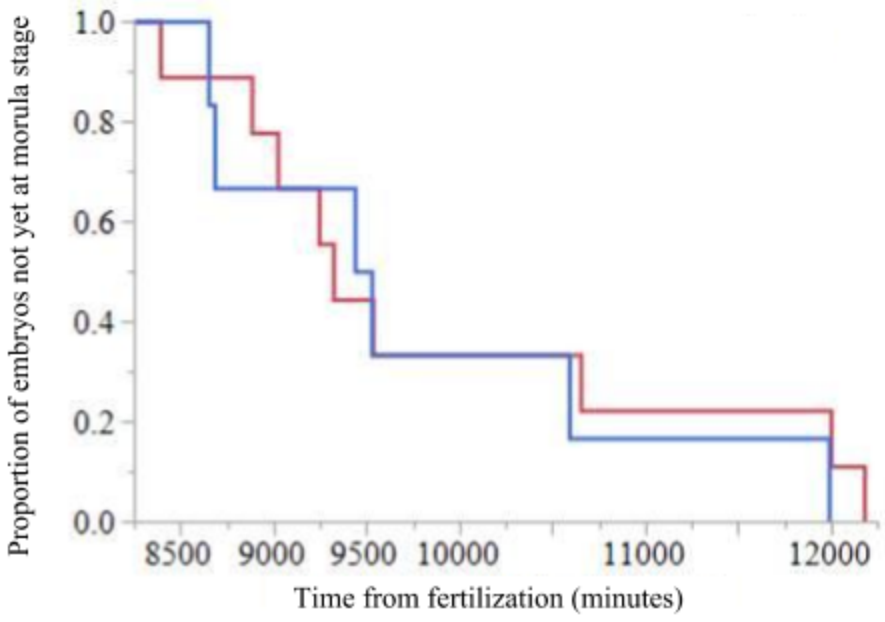


Figure 7. Kaplan-Meier Survival Analysis for development of male and female embryos to the morula stage. Male embryos are represented by the blue line, and female embryos are

represented by the red line. The y-axis represents the proportion of embryos remaining at a given time point. The x-axis represents the time in minutes. Male and female embryo development rates appear more similar and evenly distributed than they did for development to the 2-cell and 4-cell stages.

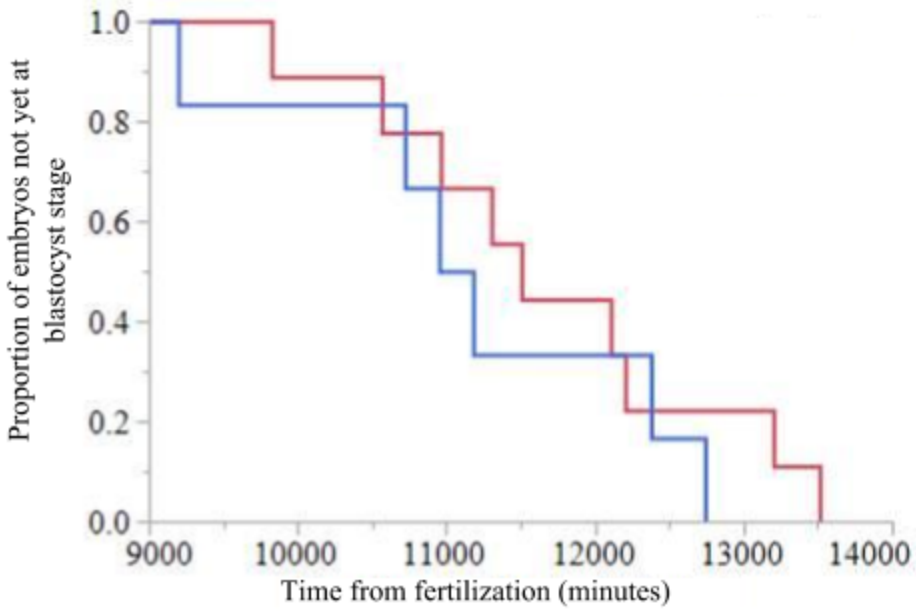


Figure 8. Kaplan-Meier Survival Analysis for development of male and female embryos to the blastocyst stage. Male embryos are represented by the blue line, and female embryos are

represented by the red line. The y-axis represents the proportion of embryos remaining at a given time point. The x-axis represents the time in minutes. Male and female embryo development rates appear more similar and evenly distributed than they did for development to the 2-cell and 4-cell stages.

DISCUSSION

Out of the 15 embryos used in this study, nine were female and six were male as determined by fluorescence microscopy. There was no difference from the expected 1:1 sex ratio during embryonic development at any stage up to the hatched blastocyst stage ($p>0.05$). Based on these results, the culture conditions used in this study do not favor the development of either sex.

I. In Vitro Embryo Growth

Only 19 embryos out of 144 developed into blastocysts during this study. This is a very low development rate compared with most other studies. There are multiple reasons why this may have occurred. First, fungal contamination affected multiple plates. This may have hindered embryo growth as the fungus could have used up nutrients in the plate before the embryos could access them. It is likely that the fungal contaminant was introduced through spores in the air in the lab. Initially, the plates were not treated with an antifungal drug, so incubating the spores would have allowed for their growth. It is also possible that the fungal contaminant was

introduced from non-sterile gloves [*e.g.* gloves that had accidentally touched skin or other non-sterile surfaces]. In order to prevent fungal growth in subsequent plates, the plates were treated with Amphotericin B (Thermo Fisher Scientific, Waltham, MA), and the outsides of each of the plates were wiped with ethanol to sterilize them. Additionally, gloves were sterilized with ethanol before handling the plates. Second, glucose is standard in our culture media even though previous studies have shown that it may affect the embryos' abilities to fully develop (26, 27). Despite the results of these studies, it is necessary to include glucose in the culture media so the embryos have a source of energy to use as they develop. The concentrations in our media may differ from those used in other studies, so the impact of glucose on embryo development in our lab may not be as severe as others have observed.

II.Quantitative Polymerase Chain Reaction (qPCR)

Despite the many months of effort towards optimizing the qPCR protocol for embryonic DNA, this method was not successful in sexing the embryos in our lab. Initially, this problem was due to the use of incorrect primers for both the autosomal gene and the Y chromosome gene. The primers initially tested were from a published source that had been cited many times specifically for the use of sexing bovine embryos. However, after many weeks of failed optimization with extremely high amplification efficiency, we performed an evaluation of the primers using the NCBI Primer-BLAST website, which showed that these primers were not specific to a single chromosome in the bovine model, let alone a single gene. It is likely that these primers were created based off of an incorrect or incomplete bovine genome, which allowed them to seem specific during those studies even though they are not in reality. Due to the non-specificity of the

original primers, we switched to primers specific for the autosomal actin housekeeping gene and the Y chromosome specific sex-determining region (SRY) gene based on the NCBI Primer-BLAST website. This greatly improved the qPCR results by reducing the range of the average quantification cycle number for both the embryo and adult samples as well as reducing the number of extraneous peaks on the melting curve graph, but this did not fully solve all the issues.

Another issue that affected the results of the qPCR trials was the use of contaminated water. The "PCR-Safe" water that was being used was a few years old and had at some point in time accidentally been contaminated. Several weeks were spent attempting to locate the source of contamination in the qPCR materials because amplification continued to occur in the non-template control wells even after replacing nearly all materials. Only after replacing the water did the non-template control contamination disappear.

Examination of the qPCR products through gel electrophoresis revealed problems and provided clues to the potential causes. The bands for the embryonic samples were at a substantially different size than the bands for the adult male Holstein control samples. This may have been the result of primer dimers due to the use of extended cycling [*i.e.* over 30 cycles]. It may also have been caused by failure to purify the amplified embryonic DNA before using it in the qPCR plates. This would have resulted in the amplification of debris, which easily could have overpowered the signal from the embryonic DNA. Sequencing of the products may show proteins from the kit or other short sequences in the genome that the primer was able to bind.

The amplification of extraneous proteins during cycling could be prevented through the use of a DNA clean-up procedure that purifies the products to only those purposefully targeted by the primers. The amplification of non-targeted proteins, which caused a difference in the size of the qPCR products, would also explain the differences in the melt curve derivative peaks. The embryo DNA samples had substantially different peaks on the melt curve derivative graphs than the adult male Holstein control DNA samples. Since the peaks did not align at the same temperature, there is a high likelihood the products were not pure but were contaminated. The different peaks indicate that the contents of the wells are different sizes. If the same primer was used in all the wells, the products all should have been of the same size. Since this was not the case, the amplification was not solely due to the primer. The next step is to submit the amplicons for sequencing to determine the sequence of the product observed on the gel, but this has not yet been completed at this time. Based on the sequence results, it may be appropriate to try a different set of primers for the autosomal actin housekeeping gene. It also may be possible that the primers are specific for one gene in the adult genome but not in the embryonic genome if the embryonic genome is not unwound completely or denatured properly before being used in the qPCR process. Either of those cases would result in a shorter genome and potentially allow the primer to bind to inappropriate, untargeted sections of the genome.

Due to the issues discussed above and the time constraints of this study, the Y chromosome-specific primer was not used in qPCR optimization trials. Since the autosomal actin housekeeping gene was not able to amplify correctly, there was little chance that the Y chromosome SRY gene would. This is because the autosomal actin housekeeping gene is found

within every embryo and thus should have been able to amplify appropriately in any and all samples. However, because this gene never amplified appropriately, it is likely that the issue lies with another component of the qPCR process, so testing the SRY gene would therefore have been futile. Further, without the autosomal gene, there would not have been a control for the qPCR trials, so false negatives during sexing would have gone unnoticed.

III. Fluorescent Sex-Y Probe Staining

Since the qPCR methods were not working well to determine the sex of the embryos, that method was replaced with staining embryos with a fluorescent probe against the Y chromosome. Initially, the only change to the protocol that came with the Bovine Sex-Y Kit was the use of whole blastocysts rather than a few biopsied cells. This was because the blastocysts were not going to be transferred to recipients after sexing, so we were willing to sacrifice whole blastocysts. However, the probe was unable to penetrate the zona pellucida of the unhatched blastocysts. To remedy this, 0.2-0.3% Triton X-100 was included along with the Y chromosome probe since Triton X-100 is able to puncture small holes in the zona pellucida. This did not improve the results of the staining though. Upon closer inspection, it became clear that many of the blastocysts were too big to properly fix to the slides, which resulted in them being lost during manipulation. In an effort to prevent this, a hybrid protocol was created with the Bovine Sex-Y Kit protocol and the embryo staining protocol commonly used in our lab for other types of stains. The hybrid protocol allowed for the use of the fluorescent probe in the Bovine Sex-Y Kit according to its intended use because the other embryo staining protocols account for the

presence of the zona pellucida and greater numbers of cells. Since the Bovine Sex-Y Kit fluorescent probe was intended to be used on only a small number of cells, the probe may have otherwise been spread too thin and only sporadically picked up its target, thereby appearing more like debris and false positives than true positive results. Further, the Bovine Sex-Y Kit fluorescent probe was not designed to be able to penetrate the zona pellucida. Failure to penetrate this layer would prevent the probe from reaching any of the embryonic DNA and thus prevent it from binding to its target. Although this new protocol was designed to work with the larger embryos containing a zona pellucida, there was still extremely limited success. Finally, the protocol was switched to include crushing the embryos on the slides before staining, thereby releasing the individual cells. This allowed them to be more easily fixed to the slides and not lost during manipulation as well as to be more easily stained by the probe. This was the final method that was used for this study. There were a few challenges even with the new protocol. One embryo was unable to be removed from the PCR tube it had been frozen in. This decreased the total blastocyst count from 19 to 18. Two additional embryos were not properly fixed to the slides during the staining process, which resulted in a total of 16 embryos to use during the final imaging.

IV. Time Lapse Video Analysis

During the time lapse video analysis, a fourth embryo was removed from the study. This embryo displayed extremely poor and abnormal development such that it was inappropriate to include this embryo in the final results. This embryo was grown in one of the plates contaminated by fungus, which may have been the cause of its irregular growth patterns. Another embryo was

already at a non-morula multi-cell stage when it was transferred to the time lapse plate. This meant that the first two cleavage divisions were unable to be assessed [*i.e.* the divisions to the 2-cell and 4-cell stages were not recorded]. A different embryo was unable to be analyzed during the cleavage division to the 2-cell stage as the embryo underwent two simultaneous cleavage divisions to transition directly from one cell to four.

V. Growth Rates in Male and Female Embryos

The results from the 15 embryos that were analyzed indicate that there is no difference in the growth rate of male and female embryos. Assessments of the average time post insemination to reach each milestone showed that male and female embryos are equally likely to reach those stages within the same amount of time. Although this work directly contradicts much of the published literature to date, it does support the conclusions of King *et al.* (32) and others (10, 19, 24, 31, 37, 47, 50, 55). Perhaps the culture conditions our lab uses to grow embryos do not favor either sex. This is difficult to conclude with certainty though since the sample size is relatively small (n=15 for this study; n=30+ for other studies).

These results do not support the hypothesis that male embryos are able to reach the blastocyst stage earlier than female embryos, which is what was initially expected. However, it is important to report results that do not match expectations or provide "exciting" results as there will otherwise be an extreme bias in the published literature (8). By reporting these results, a more accurate understanding of in vitro embryonic development can be obtained.

VI. Future Research

Others have claimed that qPCR can be used efficiently and effectively for sexing bovine embryos (36). In an effort to develop a more reliable and objective way to determine embryo sex (as opposed to fluorescence microscopy), the next steps of this research will undoubtedly include further optimization of the qPCR protocol with new primers. Developing a successful qPCR protocol would save time, energy, and resources in the future and would provide a more definitive way to determine embryo sex. Additionally, gaining a more acute understanding of the way individual culture ingredients impact embryo growth would be beneficial not only for labs using in vitro culture to study embryonic development, but this knowledge would help human infertility clinics and dairy farms create culture conditions to sway the offspring sex ratio in a controlled and desired manner. There are many new directions in which to take this research field. The impacts the results of future projects may have on the world are quite powerful.

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SUPPLEMENTAL MATERIAL

1. Holding Media Recipe

Manufacturer	Chemical Name	Preparation	Storage Temperature	Percentage of Total Volume
Gibco	Medium 199/Holding Media Base	N/A	4°C	87.75%
Corning	Fetal Bovine Serum (FBS), Heat Inactivated	Thaw	-20°C	10%
Sigma	Sodium Pyruvate	Dissolve 24.2mg in 11mL of water then filter	-20°C	1%
Sigma	Heparin for Holding Media	Dissolve 11mg in 22mL of phosphate buffered saline (PBS)	-20°C	1%
Sigma	Gentamicin	N/A	4°C	0.25%

Ingredients were combined and heated to 25°C. The pH was adjusted to 7.3-7.4 with hydrogen chloride or sodium hydroxide. The holding media was then filtered through a 22µm porous syringe filter and stored at 4°C. The holding media was stored for up to one month before replacement.

2. Oocyte Maturation Media (OMM) Recipe

Manufacturer	**Chemical Name**	**Preparation**	**Storage Temperature**	**Percentage of Total Volume**
Sigma	OMM Base	N/A	4°C	86.055%
Corning	Fetal Bovine Serum, Heat Inactivated	Thaw	-20°C	10%
Sigma	Sodium Pyruvate	Dissolve 24.2mg in 11mL of water then filter	-20°C	1%
Sigma	Gentamicin	N/A	4°C	1%
Gibco	Insulin Transferrin Selenium (ITS) 100X	N/A	4°C	1%
Gibco	Glutamax	N/A	4°C	0.5%
Sigma	Estradiol	Dilute 5mg in 5mL of ethanol	-20°C	0.2%
N/A	Folltropin-V	N/A	-20°C	0.125%
Sigma	Cysteamine	Dissolve in water	-20°C	0.1%

Corning	Epidermal Growth Factor (EGF)	Dissolve 0.1mg in 2mL of water	-20°C	0.02%

Ingredients were combined and heated to 25°C. The pH was adjusted to 7.3-7.4 with hydrogen chloride or sodium hydroxide. The oocyte maturation media was then filtered through a 22µm porous syringe filter and stored at 4°C. The oocyte maturation media was then stored for up to one month before replacement.

3. Synthetic Oviductal Fluid (SOF)-Base Media Recipe

Manufacturer	**Ingredient**	**Concentration**	**Amount**
Sigma	Sterile Water	1X	900-1000 mL
Sigma	$CaCL_2 \cdot 2H_2O$	1.17 mM	172mg
Sigma	$MgCl_2 \cdot 6H_2O$	0.49 mM	99.6mg
Sigma	KH_2PO_4	1.19 mM	161.95mg
Sigma	KCl	7.16 mM	533.8mg
Sigma	NaCl	107.7 mM	6.294g
Sigma	$NaHCO_3$	25.07 mM	2.106g
Sigma	Sodium Lactate	5.3 mM	755.5µL

The powders and sodium lactate were combined and water was added to increase the volume to 1L. The solution was then heated to 25°C and the pH adjusted to 7.1-7.2 with hydrogen chloride or sodium hydroxide. The SOF-Base media was then filtered through a 22µm porous syringe

filter and stored at 4°C. The SOF-Base media was then stored for up to six months before replacement.

4. SOF-Fert Media Recipe

Manufacturer	Chemical Name	Preparation	Storage Temperature	Percentage of Total Volume
See Supplemental Material 3	SOF-Base	N/A	4°C	87.450%
Sigma	Essentially Fatty Acid Free Albumin for SOF-Fert	Dissolve 12g of albumin in 200mL of SOF-Base	-20°C	10.000%
Sigma	Sodium Pyruvate	Dissolve 24.2mg in 11mL of water then filter	-20°C	1.000%
Sigma	100X Caffeine	Dissolve 0.3884g in 20mL of water	-20°C	1.000%
Sigma	Heparin for SOF-Fert	Dissolve 20mg in 20mL of water	-20°C	0.500%
Sigma	Gentamicin	N/A	4°C	0.050%

Ingredients were combined and heated to 25°C. The pH was adjusted to 7.3-7.4 with hydrogen chloride or sodium hydroxide. The SOF-Fert media was then filtered through a 22µm porous syringe filter and stored at 4°C. The SOF-Fert media was then stored for up to one month before replacement.

5. SOF-Bovine Embryo 1 (BE1)

Manufacturer	**Chemical Name**	**Storage**	**Preparation Method**	**Stock Concentration**	**Final Concentration**
See Supplemental Material 3	SOF-Base	4°C as a liquid	N/A	1X	1X
Sigma	Bovine Serum Albumin (BSA)	4°C as a powder (stock) -20°C as a liquid	Dissolve in SOF-Base; aliquot in 1.5mL tubes	40mg/mL	4mg/mL
Sigma	Sodium Pyruvate	4°C as a powder (stock) -20°C as a liquid	Dissolve in water; sterile filter; aliquot in 1.5mL tubes	20mM	0.4mM
Life Technologies	Essential Amino Acids 50X	4°C as a liquid	N/A	50X	1X
Life Technologies	Non-Essential Amino Acids	4°C as a liquid	N/A	100X	1X
Sigma	100X Sodium Citrate	4°C as a powder	Dissolve in water; sterile	57mM	0.57mM

		(stock) -20°C as a liquid	filter; aliquot in 1.5mL tubes		
Sigma	Myo-Inositol	4°C as a powder (stock) -20°C as a liquid	Dissolve in water; sterile filter; aliquot in 1.5mL tubes	277.5mM	2.775mM
Gibco	Insulin Transferrin Selenium (ITS) 100X	4°C as a liquid	N/A	100X	1X
Gibco	Glutamax	4°C as a liquid	N/A	100X	0.5X
Sigma	Gentamicin	4°C as a liquid	N/A	10mg/mL	25µg/mL
Corning	Epidermal Growth Factor (EGF)	4°C as a powder (stock) -20°C as a liquid	Dissolve in water; sterile filter; aliquot in PCR tubes	50µg/mL	10ng/mL

Ingredients were combined and heated to 25°C. The pH was adjusted to 7.3-7.4 with hydrogen chloride or sodium hydroxide. The SOF-BE1 media was then filtered through a 22µm porous syringe filter and stored at 4°C. The SOF-BE1 media was then stored for up to one month before replacement.

6. Bovine Housekeeping Gene (Actin) Forward Primer Sequence

The forward primer is 20 nucleotides long and only appears once within the *Bos taurus* genome on chromosome 25 according to an October 2017 search on the NCBI Primer-BLAST website. The expected size of the products was 162bp. This primer was designed only through the NCBI Primer-BLAST website search rather than through the use of a primer design software. The primer is from Thermo Fisher Scientific (Waltham, MA).

Primer Sequence: 5'-GAGCTACGAGCTTCCTGACG-3'

7. Bovine Housekeeping Gene (Actin) Reverse Primer Sequence

The reverse primer is 20 nucleotides long and only appears once within the *Bos taurus* genome on chromosome 25 according to an October 2017 search on the NCBI Primer-BLAST website. The expected size of the products was 162bp. This primer was designed only through the NCBI Primer-BLAST website search rather than through the use of a primer design software. The primer is from Thermo Fisher Scientific (Waltham, MA).

Primer Sequence: 5'-GGCAGACTTAGCCTCCAGTG-3'

8. **Table S1.** Time for male (M) and female (F) embryos to reach developmental milestones (minutes post fertilization).

Embryo ID	Sex	2-Cell	4-Cell	Morula	Blastocyst	Hatched Blastocyst
1	F	*	*	6,804	9,336	-
2	F	2,172	3,335	10,585	11,816	-
3	M	2,668	7,028	9,131	10,917	-
4	F	1,796	2,943	8,012	9,334	11,297
5	M	1,669	2,641	7,558	8,071	11,306
6	F	2,136	3,396	7,852	9,833	11,640
7	M	1,901	2,453	7,419	9,949	-
8	F	1,788	2,336	7,628	10,851	-
9	M	2,370	6,045	10,605	11,356	-
10	F	2,006	2,403	7,159	10,274	-
11	F	2,368	2,953	8,302	10,972	-
12	F	1,822	3,015	7,638	8,441	-
13	M	§	1,748	8,275	9,698	-

14	M	1,949	2,572	8,128	9,413	-
15	F	2,294	3,294	9,426	9,736	-

*This information is unavailable because the embryo was placed in the time lapse plate at a multi-cell, non-morula stage, making it impossible to observe the 2-cell and 4-cell cleavage divisions.

§This information is unavailable because the embryo appeared to divide immediately into four cells and skip the 2-cell stage division.