Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ

Melissa A. Larson*†, Koji Kimura†‡, H. Michael Kubisch§, and R. Michael Roberts¶‖

*Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110; ‡Animal Reproduction Laboratory, National Grassland Institute, Tochigi 329-2793, Japan; §Department of Veterinary Medicine, Tulane Regional Primate Research Center, Covington, LA 70433; and ¶Department of Animal Sciences, 158 ASRC, University of Missouri, Columbia, MO 65211-5300

Contributed by R. Michael Roberts, June 18, 2001

IFN-τ is a secretory product of trophectoderm of cattle, sheep, and their relatives and is expressed for a few days in early pregnancy after the blastocyst first forms. It serves to alert the mother that she is pregnant. A delayed or less than robust IFN-τ signal is a likely cause of embryonic loss. Here we have determined whether blastocyst production of IFN-τ, which is encoded by a cluster of genes on chromosome 9, differs between the sexes in cattle, as assessed by culture of *in vitro*-derived embryos on two different media, one complex (tissue culture medium 199 supplemented with serum) with coculture support, the other relatively simple (synthetic oviductal fluid plus albumin). With both media, female blastocysts produced approximately double the amount of IFN-τ as males, regardless of such variables as oocyte batch, blastocyst quality, hatching, and length of time in culture. However, in either tissue culture medium 199, which contains 5.5 mM D-glucose, or in synthetic oviductal fluid, in the presence but not in the absence of added glucose, significantly fewer female than male embryos were able to progress from the morula/early blastocyst stage to more advanced stages of development. It is possible that the differences between male and female embryos both in their production of IFN-τ and in their ability to progress in development in glucose-rich media are manifestations of phenomena that occur *in vivo* and provide plasticity in embryo selection during early pregnancy.

embryo culture | *in vitro* maturation–*in vitro* fertilization | sexual dimorphism

Gender differences among embryos can be detected well before the gonads begin to form. The most frequently reported example is in the relative rates of cleavage of male and female embryos in the first few days after fertilization. Embryos produced *in vitro* in a number of species fall into fast-cleaving and slow-cleaving groups, which are predominantly male and female, respectively. This phenomenon has been observed for bovine (1–5),** mouse (6, 7), sheep (8, 9), and human embryos (10). *In vivo*-produced male pig embryos, both before and subsequent to hatching from the zona pellucida, have also been reported to be larger and to have more cells than female embryos (11, 12). That male embryos develop faster than females is by no means universally accepted, however. Some studies have reported no differences in human (13, 14) and bovine embryos (15). Similarly, male and female pig embryos have been estimated to grow at similar rates *in utero* (16, 17).

There are at least three not mutually exclusive explanations for these contrasting observations. One, in the animal species studied, is a genetic effect resulting from the use of embryos of different breeds or strains. Another is that in cases where *in vitro* culture has been used, the culture conditions influence the outcome. For example, there have been reports that the presence of glucose in the medium may favor the growth of male bovine embryos over females.†††‡‡ A third explanation may relate to the manner in which growth rates are measured. In many cases, the end point used for *in vitro* studies has been the time taken to reach a readily observable stage in development, most usually the formation of the blastocyst. By such a standard, all embryos could have equivalent growth rates during the early cleavage stages, but the female embryos might be less capable than male embryos of making a particular developmental transition, for example, to form a blastocele or to advance from early to expanded blastocyst (EB). That failure, in turn, might be due to inadequacies of the culture medium or to other environmental stresses. There are several recent studies indicating that *in vitro*-produced male bovine embryos predominate among blastocysts and that this skew in sex ratios becomes more exaggerated at the expanded and hatched stages (18–21). Meanwhile, embryos lagging in development have been shown to be predominantly female (22). This bias toward males, which may be the outcome of *in vitro* embryo culture, although in one sense artificial, could be a reflection of an *in vivo* mechanism of the selection of male embryos. Many mammals, including some ruminants, seem to be able to adjust the sex ratio of offspring born, according to the conditions, both social and environmental, prevailing at the time of conception (23–32). In many wild populations, breeding-age females with access to the greatest nutritional resources and, in particular, energy tend to produce more male than female offspring, thereby increasing the chances of transmitting their genes to future generations (23).

An alternative mechanism for adjusting sex ratios after conception might occur if embryos of one sex signaled their presence to the mother more robustly than the other. For example, female embryos might be anticipated to have evolved methods to offset advantages conferred upon male development by the mother. The primary agent responsible for maternal recognition of pregnancy in the pecoran ruminants, such as cattle, sheep, and deer, is a type I IFN, known as IFN-τ (32, 33), which has shown some indications of dimorphic expression in red deer embryos recovered from culled hinds (29). In cattle, embryos begin to express IFN-τ as the blastocyst forms, although there is consid-

Abbreviations: EB, expanded blastocyst(s); TCM-199, tissue culture medium 199; SOF, synthetic oviductal fluid.

†M.A.L. and K.K. contributed equally to this work.

‖To whom reprint requests should be addressed. E-mail: robertsrm@missouri.edu.

Marquant-Le Guinne, B., Nibart, M., Guyader, C., Kohen, G., Esposito, L., Thuard, J. M. & Thibier, M. (1992) *Theriogenology* **37, 253 (abstr.).

††Peippo, J. & Bredbacka, P. (1996) *Theriogenology* **45,** 187 (abstr.).

‡‡Bredbacka, K. & Bredbacka, P. (1996) *Theriogenology* **45,** 191 (abstr.).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

erable variability between embryos in the amounts they produce (35–37). Here we have sought to determine whether there are gender differences in IFN-τ production among bovine embryos and whether culture conditions that can induce a bias in sex ratios at the EB stage simultaneously produce associated changes in IFN-τ expression.

Materials and Methods

Collection, Maturation, and Fertilization of Cumulus-Enclosed Oocytes. Bovine embryos were generated from oocytes aspirated from slaughterhouse-derived ovaries and matured for 24 h. Procedures for oocyte recovery and conditions for *in vitro* maturation and *in vitro* fertilization of oocytes have been described in detail (35, 36, 38, 39). The semen used throughout was a mixed pool from six different sires (Select Sires, Plains City, OH). After 18 h in fertilization medium, presumptive zygotes were washed and cumulus cells were removed.

Embryo Culture. In Experiment 1, groups of 25 presumptive zygotes were placed in 25-μl drops of Chatot–Ziomek–Barister (CZB) medium without glucose (36, 39) overlaid with mineral oil (Sigma and Fisher Scientific) and cultured for 48 h at 39°C. Embryos that had reached the eight-cell stage or beyond were then selected and placed 25 per drop into 25-μl drops of tissue culture medium 199 (TCM-199) (which contains 5.5 mM D-glucose), supplemented with 10% FBS on a layer of buffalo rat liver cells. EB (blastocele cavity fully developed, embryo diameter slightly increased, and zona pellucida showing signs of thinning) first began to form on day 7 after insemination (in vitro fertilization = day 0). Beginning on the evening of day 7 and every 12 h thereafter until the morning of day 10, EB were removed from the pool of developing embryos and cultured individually in 50-μl drops of TCM-199 in the presence of buffalo rat liver cells. The medium was collected and replaced every 24 h. EB that appeared on the evening of day 7 were, therefore, in culture for three consecutive 24-h periods, and those present on the evenings of days 8 and 9 were cultured for 48 h and 24 h, respectively. Likewise, EB that appeared on the mornings of days 8, 9, and 10 were also cultured for 72 h, 48 h, and 24 h, respectively. The experiment was repeated on four separate sets of embryos. A preceding study, not reported here in detail, used six different collections of ovaries for oocyte collection.

In Experiment 2, the same culture conditions were used, except that embryos were withdrawn from the pools on the mornings of days 3, 6, and 8 for sex determination. This experiment was repeated eight times, but results were combined for statistical purposes.

In Experiment 3, conditions for *in vitro* maturation and in vitro fertilization were standard, but zygotes were placed immediately in modified synthetic oviductal fluid (SOF) medium (40) lacking vitamins and either with or without glucose (5.5 mM). The medium under both sets of conditions was supplemented with BSA (5 mg/ml) rather than FBS. No coculture support was used. As in Experiment 2 above, embryos were removed for sex determination on days 3, 6, and 8. This experiment was repeated 12 times, and values again were pooled by day for the two treatments.

In Experiment 4, *in vitro* maturation and in vitro fertilization were standard, and resulting zygotes were cultured in SOF without glucose until day 8, when EB were selected and placed in individual microdrop cultures as described in Experiment 1 above. At 24 h and 48 h, the medium was removed for assay of IFN-τ, and the sex of each blastocyst was determined.

Quality and Hatching Status of Blastocysts. EB were given a quality score based on morphological assessment (1, embryos free of fragmented cells; 2, some granulation and initial signs of fragmentation; 3, collapsed or badly fragmented). A "stage of hatching" score was assigned to each EB at the time it was harvested (1, embryo had not hatched; 2, blastocyst in the process of hatching; 3, embryo no longer enclosed by the zona pellucida).

Analysis of IFN Content by Antiviral Assay. The IFN-τ content of the medium was measured by a cytopathic reduction assay in Madin–Darby bovine kidney (MDBK) cells challenged by vesicular stomatitis virus (35, 36). The culture medium was assayed in a series of increasing 3-fold dilutions. After the viral challenge, cells were fixed in methanol for ≈5 min, air dried, and stained with 0.5% gentian violet. Because the volume of medium available was small (50 μl), each sample could be assayed only once. The standard included on each plate was recombinant bovine IFN-τ1a (41) with an antiviral activity (the concentration at which it protected 50% of a monolayer of MDBK cells) of 0.97 pM. It was assumed that the activity of this recombinant product ($M_r \approx 18{,}000$) was similar to that of the glycosylated boIFN-τ ($M_r \approx 20{,}000$) produced by the blastocysts.

Sexing of Blastocysts. EB were extracted at 55°C for 30 min in 5 μl of lysis buffer (20 mM Tris·HCl/0.9% Nonidet P-40/0.9% Tween 20/0.4 mg/ml proteinase K) (42) and proteinase denatured by heating to 98°C for 10 min. Sexing was then performed by PCR amplification of a bovine Y-specific DNA sequence as described by Peura *et al.* (43). Non-sex-specific bovine satellite primers were always included in the reaction to demonstrate the presence of embryo DNA (43). Bovine male genomic DNA, bovine female genomic DNA, and water samples were included as controls and treated in the same manner as blastocyst samples. The EB extracts were adjusted to an annealing temperature of 62°C, and the PCR reaction mix, consisting of PC2 buffer (Ab Peptides, St. Louis), 100 μm dNTPs, 0.25 pM satellite primers, 0.5 pM Y primers, and 2.5 units of Klentaq with Pfu DNA polymerase (Ab Peptides and Stratagene), was added to bring the final volume to 20 μl. After 30 rounds of amplification (44), PCR products were visualized by electrophoresis on a 1% agarose gel (45). Amplification of bovine DNA by satellite oligonucleotides yielded a 216-bp band, and the Y-specific oligonucleotides yielded a 301-bp band.

Statistical Analyses. IFN-τ production was highly variable, and the variance between means was heterogeneous. Raw IFN values were therefore log-transformed to reduce the variability between samples. Log-transformed means were analyzed by the General Linear Models procedure of SAS (SAS Institute, Cary, NC). The statistical model included replicate, sex, day of blastocyst formation, quality score, hatching score, and time in culture. All interactions of the above main effects were also analyzed. Differences between male and female blastocysts were also analyzed with the General Linear Models procedure. The statistical model included quality score, hatching score, time in culture, and day of blastocyst formation. Unless stated otherwise, values are reported as least squares means with their actual standard error. Sex ratios were compared with an expected 50:50 ratio by a corrected χ^2 procedure (table 4.13 in ref. 46).

Results

Preliminary Experiments. Several experiments were carried out under the culture conditions used for Experiment 1 but are not reported in detail. They showed that the sex of individual blastocysts could be reliably determined, and that the presence of sperm, which might be present in the zona pellucida, did not interfere with the determination of sex. No amplification of Y-chromosome sequence was evident when up to 1,000 sperm were included in the PCR reaction, although the satellite band was evident at this concentration. The experiments also confirmed that EB first began to appear toward the end of day 7 and

that the mean time for EB to form was 8.5 ± 0.1 days and 8.6 ± 0.1 days for male and female embryos, respectively. Finally, the experiments showed that IFN-τ could only be reliably measured in EB forming on day 7, provided they were cultured for 24 h rather than for shorter periods.

Experiment 1: Determining Whether IFN-τ Production Is a Sexually Dimorphic Feature at Blastocyst. In this experiment, a total of 1,289 presumptive zygotes were used in the four replicates. The number that cleaved was 88.9%, and a total of 276 (21.4%) of the fertilized oocytes developed to EB, of which 269 were cultured individually from four replicates (n = 92, 55, 67, and 55, respectively). EB were first detected on day 7 and continued to appear until day 10. The last samples were therefore collected and blastocysts frozen on day 11. All blastocysts were sexed, and medium samples (625 in all) were assayed together after the fourth replicate had been conducted.

Analysis of the factors affecting IFN-τ production was conducted for five variables: replicate, quality score, hatching score, day of EB formation, and length of time in culture. IFN-τ production differed by replicate (Fig. 1*A*). In this experiment, in which the oocytes were all collected in June 2000, the replicate values were less variable than in an earlier experiment of similar design (data not shown) conducted in February and March. The quality of the blastocysts had no effect on mean IFN-τ production (781 ± 53, 697 ± 59, and 563 ± 74 pg for quality scores 1, 2, and 3, respectively; P = 0.2), and blastocysts that hatched produced more IFN-τ than those that did not hatch (Fig. 1*C*). EB that formed on different days did not differ significantly in their mean IFN-τ production when the first 24-h period of culture was analyzed (P = 0.06), although there was a tendency for late-developing EB to produce more IFN than early ones (Fig. 1*B*).

The 269 blastocysts examined in Experiment 1 displayed a bias in the sex ratio comparable to that observed in two preliminary studies. Male EB outnumbered females 171 to 98 (0.64:0.36; $P <$ 0.01). In the two preliminary experiments the values were 0.71:0.29 ($P < 0.05$; n = 31) and 0.66:0.34 ($P < 0.01$; n = 235), respectively. No difference was detected in Experiment 1 on the mean day of EB formation between the sexes (8.4 ± 0.1 days for both males and females; P = 0.5). Male EB were scored at higher quality than females in Experiment 1 (quality score 1.4 vs. 1.7, respectively; $P < 0.001$), and males were more likely to hatch in culture than females (hatching score 1.7 vs. 1.4, respectively; $P <$ 0.001).

Significantly, female EB produced almost twice as much IFN-τ as males when all samples were combined for analysis (883 ± 82 vs. 478 ± 32 pg per embryo, respectively; $P < 0.001$). In addition, IFN-τ production by females consistently exceeded that of males in all four replicates (Fig. 2*A*). When the data were analyzed by hour of culture, female EB secreted more IFN-τ than males at each time point (24 h, 48 h, and 72 h; Fig. 2*B*).

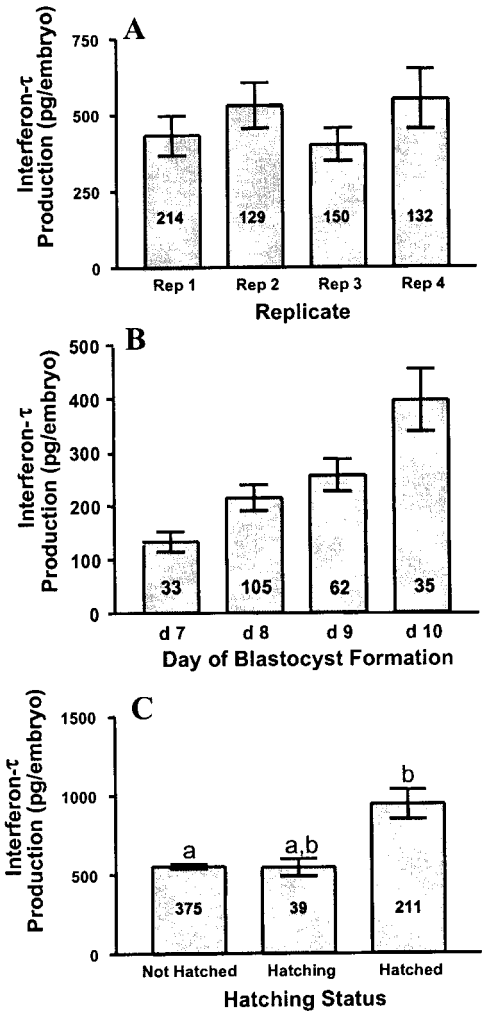

Fig. 1. IFN-τ production by EB in Experiment 1. (*A*) Total mean production of IFN-τ for each replicate (oocyte collection). All values for IFN-τ within a replicate were combined for analysis. Values shown are least-squares means. IFN-τ production differed by replicate ($P < 0.01$). The number of samples in each replicate is shown within each column. (*B*) Mean IFN-τ production for the first 24 h of culture for blastocysts forming on days 7–10. Although the day of blastocyst formation was not a significant effect in the statistical model (P = 0.06), there was a trend for earlier forming blastocysts to produce less IFN-τ. The number of samples is shown within columns. (*C*) Increase in mean production of IFN-τ associated with blastocyst hatching. Different superscripts indicate that means differ ($P < 0.01$). The number of samples is shown within columns.

Experiment 2: Determining When in Development the Sex Ratio Bias First Occurs. Seven replicates were performed with a total of 3,859 inseminated oocytes, of which 74.2% cleaved. Embryos were removed from group cultures on the mornings of days 3, 6, and 8 to determine the stage at which the sex ratio became skewed toward males. All replicates showed the same trends, although numbers within replicates were insufficient for robust statistical analysis. Therefore, all values across replicates were combined. At day 8, the EB that had formed were carefully classified as either early (first signs of a blastocele), blastocyst (obvious blastocele), late (blastocele either fully or close to fully formed, but no increase in diameter of the embryo), or expanded. At time of selection, embryos were frozen for sexing. Despite the rather subjective manner in which the blastocysts were classified, the data are quite clear-cut (Table 1). A sex ratio close to 1:1 was maintained until the embryos reached the early blastocyst stage. Moreover, there was no evidence that males advanced in development faster than females until day 6. Morulae at day 8 were predominantly female, whereas the majority of day 8 blastocysts, other than those in the very earliest stages of development, were male. Males at the EB stage at day 8 markedly outnumbered females (ratio 0.84:0.16).

Experiment 3: Determining the Effects on Sex Ratio of Glucose in the Medium. In this experiment, SOF medium either with or without glucose (5.5 mM) was used for embryo culture from the time of insemination until day 8, when the experiment was terminated, to determine whether the presence of the sugar influenced the sex ratio at EB (Table 2). Twelve batches of oocytes were used to minimize the effect of replicate. Cleavage of fertilized oocytes and progression to the eight-cell stage by the morning of day 3 were comparable for zygotes cultured on SOF in either the

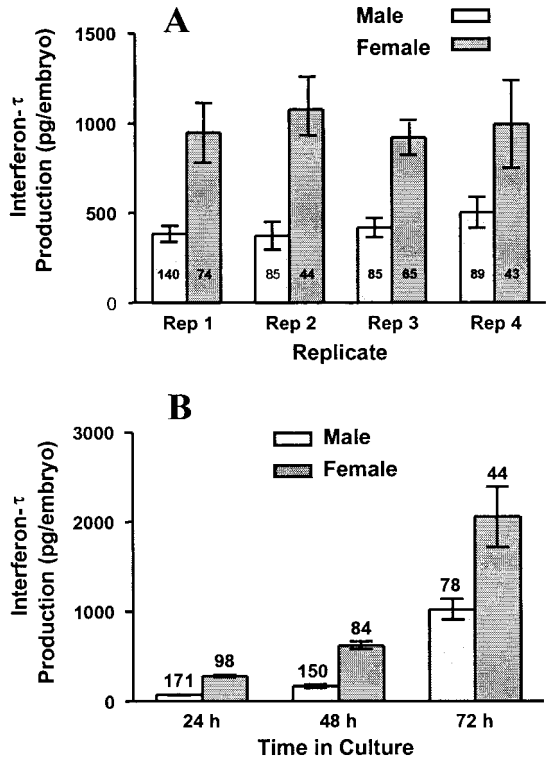

Fig. 2. Differences between the sexes in mean production of IFN-τ in Experiment 1. (*A*) Total production of IFN-τ by male and female EB for each replicate. Means differed significantly in all four replicates ($P < 0.01$). The number of samples is shown within columns. (*B*) Differences in IFN-τ production between the sexes according to time spent in culture. Means for male and female EB differ significantly for each culture time ($P = 0.02$). The number of samples is shown above the columns.

presence or absence of glucose (Table 2). However, whereas 69% of eight-cell embryos in glucose-free medium reached the EB stage by day 8, the corresponding value for eight-cell embryos cultured in glucose-containing medium was less than half that. In the presence of glucose, the sex ratio began to diverge at the early blastocyst stage, so that at EB more than 75% of the embryos were male. In contrast, the sex ratio did not deviate significantly from 1:1 throughout development in glucose-free SOF (Table 2).

Experiment 4: Determining Whether Sexual Dimorphism in IFN-τ Production Is Maintained Among Blastocysts Where the Sex Ratio Is Not Skewed. This study was designed to determine whether the difference in IFN-τ production noted between males and females was still evident in the EB that formed during culture on SOF medium in the absence of glucose (see Experiment 3). A total of 366 oocytes were inseminated from three batches of ovaries. Of these, 160 had reached the eight-cell stage by the morning of day 3, and 110 reached EB by the morning of day 8. At this stage, a total of 89 EB were placed in individual microdrop cultures. At 24 h, and again at 48 h, the medium was removed for assay of IFN. Female and male EB at the termination of the experiment numbered 45 and 44, respectively. Initial quality scores were 1.33 ± 0.11 (females) and 1.39 ± 0.11 (males). By 48 h, the quality of the embryos had deteriorated (females, 1.96 ± 0.18; males 2.27 ± 0.18). Differences in IFN-τ production were evident between the sexes after 24 h of culture (females, 99.4 ± 13.3 pg per embryo; males, 53.7 ± 6.9 pg per embryo; $P < 0.05$) and after 48 h (females 125.4 ± 13.3 pg per embryo; males 73.0 ± 9.7 pg per embryo; $P < 0.01$). In contrast to culture in supplemented TCM-199 (Experiments 1 and 2), few of the EB seemed capable of hatching, suggesting that additional factors, possibly supplied by either serum or the buffalo rat liver cells used for coculture on TCM-199, are needed for further development once the embryos reach the EB stage. These data show that the sexual dimorphism in IFN-τ production persists during culture on a medium that does not cause a skew in sex ratio.

Table 1. Development and sex ratio of bovine embryos after different times in culture in TCM-199

Collection stage	Day	Embryo number	Sex Ratio, male:female
Eight-cell	3	192	46:54
Morula	6	153	50:50
Morula	8	103	37:63*
Early blastocyst	8	79	52:48
Blastocyst	8	72	67:33*
Late blastocyst	8	42	64:36
Expanded blastocyst	8	65	83:17†

*Significantly different from anticipated 1:1 ratio of males to females ($P < 0.05$).
†Significantly different from anticipated 1:1 ratio of males to females ($P < 0.01$).

Discussion

In this study we show that in two different but widely used media, one relatively simple (SOF), the other highly complex (TCM-199 with serum supplementation and coculture support), no differences could be observed in the rate at which male and female embryos developed to morulae. Even the average time taken for male and female embryos to reach the EB stage in TCM-199 was the same (8.5 days), although far fewer female morulae actually made the transition from morula to EB. Our results, therefore, support those of others (15) that *in vitro*-produced male bovine embryos do not have an inherently faster rate of growth than females.

Others have noted a bias in the sex ratio toward males in populations of bovine embryos cultured to the EB stage, although the magnitude of the effect has been variable (4, 5, 21–22),[§§¶¶] and its basis is not well understood. In our experiments, the phenomenon clearly results from a failure of female embryos cultured on the complex TCM-199 medium to make the transition to EB rather than from the faster growth of males. As suspected by Carvalho *et al.* (22) and Gutierrez-Adan *et al.* (47), glucose may be the reagent in the medium that is deleterious to female embryo development. Our data (Table 2) confirmed that a simple, glucose-free medium allowed female embryos to form EB as easily as males, and that the addition of 5.5 mM glucose to this medium was selectively detrimental to female EB formation. The data probably explain why male embryos have been reported to grow more rapidly in the presence of glucose than female embryos when attaining the blastocyst stage has been used as the end point. Whether lower glucose concentrations or the substitution of some other sugar, such as D-fructose, would be less damaging to female embryos remains unclear, although our preliminary data (K.K. and R.M.R., unpublished results) show that development to the EB stage is as efficient in SOF with D-fructose as it is in its absence.

Embryos become more dependent on aerobic metabolism and probably acquire a greater capacity to use glucose as the blastocyst forms (48–50), but there are significant differences

[§§]Peippo, J. & Bredbacka, P. (1996) *Theriogenology* **45,** 187 (abstr.).

[¶¶]Bredbacka, K. & Bredbacka, P. (1996) *Theriogenology* **45,** 191 (abstr.).

Table 2. The effect of D-glucose on development and sex ratio of bovine embryos cultured in SOF medium

		5.5 mM glucose		No glucose	
Collection stage	Day	Embryo number	Males (%)	Embryo number	Males (%)
Eight-cell	3	129	65 (50.4)	129	61 (47.3)
Morula	6	117	69 (59.0)	121	60 (49.6)
Morula	8	73	43 (58.9)	40	21 (52.5)
Early blastocyst	8	86	58 (67.4)*	46	27 (58.7)
Blastocyst	8	86	58 (67.4)*	81	43 (53.1)†
Late blastocyst	8	44	30 (68.2)	87	55 (63.2)
Expanded blastocyst	8	49	37 (75.5)*	189	99 (52.4)†

On the SOF medium containing glucose, 82.3% of the fertilized oocytes cleaved and 50.9% had reached the eight-cell stage by the morning of day 3. The corresponding values for SOF without glucose were 84.7% and 55.0%, respectively.
*Significantly different from anticipated 1:1 ratio of males to females ($P < 0.05$).
†Significantly different when compared with male-to-female ratio at 5.5 mM glucose ($P < 0.05$).

between *in vitro*- and *in vivo*-produced embryos in the manner and rate at which they metabolize such carbohydrates. In particular, the pentose phosphate pathway seems more active in *in vitro*-produced bovine blastocysts (48, 51). *In vitro* culture also appears to lead to delayed X chromosome inactivation in female mammalian embryos (44, 52) and to an increase in the mRNA for glucose 6-phosphate dehydrogenase and for other X-linked genes (47). Inactivation of the paternal X chromosome occurs in trophectoderm of female murine embryos at about the time of blastocele formation, whereas random X inactivation in the embryo proper occurs later (53, 54). Therefore, any deleterious effect of a double dose of X chromosome among blastocysts is likely to be manifested in trophectoderm selectively. Glucose 6-phosphate dehydrogenase is the rate-limiting enzyme for the pentose phosphate pathway, but whether overexpression of this enzyme and the associated increased carbon flow through the pathway in emerging trophectoderm of female embryos is an explanation for the general failure of females to advance to the EB stage in the presence of glucose is unclear. It is similarly uncertain whether growth in glucose-containing medium is the cause of the delay in X inactivation. Interestingly, embryo transfer performed with blastocysts, rather than embryos at earlier stages, leads inadvertently to a disproportionate number of males born in both cattle (55) and humans (56–58).

Gutierrez-Adan *et al.* (47) hypothesized that sexually dimorphic gene expression, such as that observed for glucose 6-phosphate dehydrogenase, may provide a means of controlling sex ratio in wild animal populations, by favoring the growth rate of male embryos over female embryos. Although our data suggest that growth rates of male and female embryos in culture are not different, a selective block to development, operating preferentially against one sex, would achieve the same purpose. Glucose concentrations within the female reproductive tract of several mammals are much lower than those in serum immediately after ovulation (refs. 59 and 60 and references therein) but increase as progesterone concentrations rise (61). In species that carry a single embryo, an enriched uterine environment, possibly brought about by an advanced luteal phase, as noted in dominant hinds (29), might ensure that male embryos are more likely to establish a pregnancy than females. In litter-bearing species, similar conditions might lead to selective preimplantation loss of females within a litter. Consistent with this prediction, a recent study performed on fallow deer has shown that provision of a high dietary energy ration to improve the body condition of young hinds increased the percentage of male calves born from 46% to 75% and that the increase was positively correlated with the concentration of insulin-like growth factor 1 in the serum of the mothers (62). Similarly, when large cattle data sets have been analyzed, the overall percentage of male calves born averaged only slightly above 50% (63). However, the sex ratio of males to females became significantly greater than 1:1 in herds where the nutritional status of the cows was high, and more male than female calves were born to heifers at a high level of nutrition at the time of conception and early pregnancy.

The sexually dimorphic difference in IFN-τ production, with female EB producing almost twice as much IFN as males, was unanticipated, because the eight or more actively transcribed bovine IFN-τ genes are clustered on chromosome 9 and were not associated with the X chromosome (41). A number of possible nonepigenetic explanations must, therefore, be considered to explain the higher expression of IFN-τ in females. One is embryo quality, because quality 1 EB produce rather more IFN-τ than blastocysts classified as quality 2 or 3, but as male EB in experiment 1 were, on average, of higher quality than female blastocysts, this explanation is not tenable. Another possible basis for a sex difference is the extent of blastocyst hatching, as hatched and hatching blastocysts produced more IFN-τ than zona-enclosed EB (Fig. 1*C*). Again, however, males predominated among the EB that hatched. The length of time an EB remains in culture also influences how much IFN is produced (Fig. 2*B*), but a retrospective analysis of the data in Experiment 1 (and preceding experiments not reported) revealed no differences either in the length of time male and female EB remained in culture or in the day on which males and females reached the EB stage. The question of cell numbers was not directly addressed in the present study and hence cannot be eliminated as the possible basis of male/female differences in IFN-τ production. Nevertheless, in earlier experiments performed under culture conditions identical to those used in Experiment 1, there was no correlation between cell number and IFN-τ released into the medium for blastocysts of the same age (36, 38, 64), and, wherever cell number and sex of embryos have been determined, male blastocysts tend to contain more cells than females (9, 12, 18, 65, 66). Consequently, we believe that the differences in IFN-τ production between males and females are due to differential gene expression between the sexes.

It seems unlikely that overexpression of IFN-τ by female EB is a stress response arising from the different abilities of male and female embryos to tolerate the presence of glucose in the medium, because the effect persisted in embryos produced in SOF medium, where both sexes developed equally well. A hint that sexually dimorphic expression of IFN-τ might occur in other ruminant species arose from the studies of Flint *et al.* (29), who noted that antiviral activity was present in the uterine flushings of culled red deer hinds carrying female but not male conceptuses. To explain why dominant red deer hinds deliver predominantly male offspring, the authors proposed that male blastocysts produce IFN-τ earlier than female blastocysts, which provides them with an advantage in

establishing a pregnancy in dominant hinds. We favor another theory, namely that female embryos can signal their presence more robustly than males, thereby offsetting the advantages male embryos might have to survive the transition to blastocyst in an enriched nutritional environment. Although it is unclear whether the differences noted in our experiments between male and female bovine embryos during their development in culture are manifestations of phenomena that occur naturally *in vivo*, they could potentially provide plasticity in embryo selection during the establishment pregnancy.

We thank George Seidel, Colorado State University, and Neal First, University of Wisconsin–Madison, for critical reading of the manuscript, Alan Ealy for help with the statistical analysis, and Jon Green for discussion. Lee Spate provided technical assistance, and Select Sires donated the bull semen. Severence McLaughlin did some of the preliminary sex determinations on blastocysts cultured in TCM-199. The research was supported by grants from the National Institutes of Health (HD 21896 to R.M.R. and HD 36421 to H.M.K.) and the United States Department of Agriculture (NRI 96-35205-3766 to R.M.R.). K.K. was supported by a "Research Abroad" Grant from the Science and Technology Agency of Japan.

1. Avery, B. (1989) *J. Reprod. Fertil.* **87,** 627–631.
2. Avery, B., Madison, V. & Greve, T. (1991) *Theriogenology* **35,** 953–963.
3. Avery, B., Jorgenson, C. B., Madison, V. & Greve, T. (1992) *Mol. Reprod. Dev.* **32,** 265–270.
4. Yadav, B. R., King, W. A. & Betteridge, K. J. (1993) *Mol. Reprod. Dev.* **36,** 434–439.
5. Beyhan, Z., Johnson, L. A. & First, N. L. (1999) *Theriogenology* **52,** 35–43.
6. Valdivia, R. P., Kunieda, T., Azuma, S. & Toyoda, Y. (1993) *Mol. Reprod. Dev.* **35,** 121–126.
7. Peippo, J. & Bredbacka, P. (1995) *Mol. Reprod. Dev.* **40,** 56–61.
8. Bernardi, M. L. & Delouis, C. (1996) *Hum. Reprod.* **11,** 621–626.
9. Catt, S. L., O'Brien, K., Maxwell, W. M. C. & Evans, G. (1997) *Theriogenology* **48,** 1369–1378.
10. Pergament E., Fiddler, M., Cho, N., Johnson, D. & Holmgren, W. J. (1994) *Hum. Reprod.* **9,** 1730–1732.
11. Cassar, G., King, W. A. & King, G. J. (1994) *J. Reprod. Fertil.* **101,** 317–320.
12. Cassar, G., de la Fuente, R., Yu, Z., King, G. J. & King, W. A. (1995) *Theriogenology* **44,** 879–884.
13. Ng, E., Claman, P., Leveille, M. C., Tanphaichitr, N., Compitak, K., Suwajanakorn, S. & Wells, G. (1995) *J. Assist. Reprod. Genet.* **12,** 566–568.
14. Munne, S., Tang, Y. X., Weier, H. U., Stein, J., Finkelstein, M., Grifo, J. & Cohen, J. (1993) *Zygote* **1,** 155–162.
15. Holm, P., Shukri, N. N., Vatja, G., Booth, P., Bendixen, C. & Callesen, H. (1998) *Theriogenology* **50,** 1285–1299.
16. Kaminski, M. A., Ford, S. P., Youngs, C. R. & Conley, A. J. (1996) *J. Reprod. Fertil.* **106,** 107–110.
17. Pomp, D., Good, B. A., Geisert, R. D., Corbin, C. J. & Conley, A. J. (1995) *J. Anim. Sci.* **73,** 1408–1415.
18. Xu, K. P., Yadav, B. R., King, W. A. & Betteridge, K. J. (1992) *Mol. Reprod. Dev.* **31,** 249–252.
19. Grisart, B., Massip, A., Collette, L. & Dessy, F. (1995) *Theriogenology* **43,** 1097–1106.
20. Pegoraro, L. M. C., Thuard, J. M., Delalleau, N., Guerin, B., Deschamps, J. C., Marquant-Le Guienne, B. & Humblot, P. (1998) *Theriogenology* **49,** 1579–1590.
21. Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P. & Boland, M. P. (1999) *J. Reprod. Fertil.* **117,** 159–167.
22. Carvalho, R. V., Del Campo, M. R., Palasz, A. T., Plante, Y. & Mapletoft, R. J. (1996) *Theriogenology* **45,** 489–498.
23. Trivers, R. L. & Willard, D. E. (1973) *Science* **179,** 90–92.
24. Rutberg, A. T. (1986) *Am. Nat.* **127,** 89–94.
25. Kojola, I. & Eloranta, E. (1989) *Evolution (Lawrence, Kans.)* **43,** 1331–1336.
26. Kent, J. P. (1995) *Behav. Ecol. Sociobiol.* **36,** 101–104.
27. Wauters, L. A., de Crombrugghe, S. A., Nour, N. & Matthysen, E. (1995) *Behav. Ecol. Sociobiol.* **37,** 189–193.
28. Cassinello, J. (1996) *Behav. Ecol. Sociobiol.* **38,** 417–424.
29. Flint, A. P. F., Albon, S. D. & Jafar, S. I. (1997) *Gen. Comp. Endocrinol.* **106,** 374–383.
30. Krackow, S. (1990) *J. Exp. Zool.* **256,** 106–112.
31. Krackow, S. (1997) *J. Reprod. Fertil.* **110,** 87–90.
32. Krackow, S. & Burgoyne, P. S. (1997) *Physiol. Behav.* **63,** 81–84.
33. Roberts, R. M., Cross, J. C. & Douglas, W. L. (1992) *Endocr. Rev.* **13,** 432–452.
34. Demmers, K. J., Derecka, K. & Flint, A. (2001) *Reproduction* **121,** 41–49.
35. Hernandez-Ledezma, J. J., Sikes, J. D., Murphy, C. N., Watson, A. J., Schultz, G. A. & Roberts, R. M. (1992) *Biol. Reprod.* **47,** 374–380.
36. Kubisch, H. M., Larson, M. A. & Roberts, R. M. (1998) *Mol. Reprod. Dev.* **49,** 254–260.
37. Larson, M. A. & Kubisch, H. M. (1999) *Hum. Reprod.* **14,** 2075–2079.
38. Kubisch, H. M., Larson, M. A., Ealy, A. D., Murphy, C. N. & Roberts, R. M. (2001) *Anim. Reprod. Sci.* **66,** 1–13.
39. Hernandez-Ledezma, J. J., Villanueva, C., Sikes, J. D. & Roberts, R. M. (1993) *Theriogenology* **39,** 1267–1277.
40. Takahashi, Y. & First, N. L. (1992) *Theriogenology.* **37,** 963–978.
41. Ealy, A. D., Larson, S. F., Liu, L., Alexenko, A. P., Winkleman, G. L., Kubisch, H. M., Bixby, J. A. & Roberts, R. M. (2001) *Endocrinology* **142,** 2906–2915.
42. Canseco, R. S., Sparks, A. E. T., Page, R. L. & Russell, C. G. (1994) *Transgenic Res.* **3,** 20–325.
43. Peura, T., Hyttinen, M., Turunen, M. & Janne, J. (1991) *Theriogenology* **35,** 547–555.
44. De La Fuente, R., Hahnel, A., Basrur, P. K. & King, W. A. (1999) *Biol. Reprod.* **60,** 769–775.
45. Voytas, D. (2000) in *Current Protocols in Molecular Biology*, eds. Ausubel, R. B. F. M., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), pp. 2.5A.1–2.5A.9.
46. Snedecor, G. W. & Cochran, W. G. (1967) *Statistical Methods* (Iowa State Univ. Press, Ames), 6th Ed.
47. Gutierrez-Adan, A., Oter, M., Martinez-Madrid, B., Pintado, B. & De La Fuente, J. (2000) *Mol. Reprod. Dev.* **55,** 146–151.
48. Rieger, D. (1992) *Theriogenology* **37,** 75–93.
49. Donnay, I., Partridge, R. J. & Leese, H. J. (1999) *Reprod. Nutr. Dev.* **39,** 523–533.
50. Donnay, I. & Leese, H. J. (1999) *Mol. Reprod. Dev.* **53,** 171–178.
51. Rieger, D., Loskutoff, N. M. & Betteridge, K. J. (1992) *J. Reprod. Fertil.* **95,** 585–595.
52. Latham, K. E. (1996) *Trends Genet.* **12,** 134–138.
53. Clerc, P. & Avner, P. (2000) *Science* **290,** 1518–1519.
54. Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W., Yanagamachi, R. & Jaenisch, R. (2000) *Science* **290,** 1578–1581.
55. Van Wagtendonk-de Leeuw, A. M., Aerts, B. J. & den Daas, J. H. (1998) *Theriogenology* **49,** 883–894.
56. Tarin, J. J., Bernabeu, R., Baviera, A., Bonada, M. & Cano, C. (1995) *Hum. Reprod.* **10,** 2992–2998.
57. Quintans, C. J., Donaldson, M. J., Blanco, L. A. & Pasqualini, R. S. (1998) *J. Assist. Reprod. Genet.* **15,** 403–404.
58. Menezo, Y. J. R., Chouteau, J., Torello, M. J., Girard, A. & Veiga, A. (1999) *Fertil. Steril.* **72,** 221–224.
59. Leese, H. J., Tay, J. I., Reischl, J. & Downing, S. J. (2001) *Reproduction* **121,** 139–346.
60. Ludwig, T. E., Lane, M. & Bavister, B. D. (2001) *Biol. Reprod.* **64,** 1366–1374.
61. Gardner, D. K., Lane, M., Calderon, I. & Leeton, J. (1996) *Fertil. Steril.* **65,** 349–353.
62. Enright, W. J., Spicer, L. J., Kelly, M., Culleton, N. & Prendiville, D. J. (2001) *Small Ruminant Res.* **39,** 253–259.
63. Skjervold, H. & James, W. J. (1979) *Z. Tierzüchtg. Züchtgsbiol.* **95,** 293–305.
64. Kubisch, H. M., Larson, M. A. & Kiesling, D. O. (2001) *Mol. Reprod. Dev.* **58,** 390–397.
65. Burgoyne, P. S. (1993) *Development (Cambridge, U.K.)* **117,** 341–345.
66. Ray, P. F., Conaghan, J., Winston, R. M. L. & Handyside, A. H. (1995) *J. Reprod. Fertil.* **104,** 165–171.