UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA
DEPARTAMENTO DE FISIOLOGÍA (FISIOLOGÍA ANIMAL)

TESIS DOCTORAL

SEX RATIO CONTROL BEFORE FERTILIZATION AND PREIMPLANTATIONAL SEXUAL DIMORPHISM IN BOVINE

CONTROL DE LA PROPORCIÓN DE SEXOS ANTERIOR A LA FECUNDACIÓN Y DIMORFISMO SEXUAL PREIMPLANTACIONAL EN LA ESPECIE BOVINA

TESIS DOCTORAL

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Pablo Bermejo Álvarez

Directores:

Alfonso Gutiérrez Adán, Pedro Luis Lorenzo González, Dimitrios Rizos

Madrid, 2010

ISBN: **978-84-693-8306-3**

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

SEX RATIO CONTROL BEFORE FERTILIZATION AND PREIMPLANTATIONAL SEXUAL DIMOPHISM IN BOVINE

CONTROL DE LA PROPORCIÓN DE SEXOS ANTERIOR A LA FECUNDACION Y DIMORFISMO SEXUAL PREIMPLANTACIONAL EN LA ESPECIE BOVINA

TESIS DOCTORAL

PABLO BERMEJO ÁLVAREZ

Madrid, 2010

SEX RATIO CONTROL BEFORE FERTILIZATION AND PREIMPLANTATIONAL SEXUAL DIMORPHISM IN BOVINE

CONTROL DE LA PROPORCIÓN DE SEXOS ANTERIOR A LA FECUNDACIÓN Y DIMORFISMO SEXUAL PREIMPLANTACIONAL EN LA ESPECIE BOVINA

Pablo Bermejo Álvarez

Directors/Directores: Alfonso Gutiérrez Adán

Pedro Luis Lorenzo González

Dimitrios Rizos

Los doctores Alfonso Gutiérrez Adán y Dimitrios Rizos, Investigadores Titulares del Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), y el doctor Pedro Luis Lorenzo González, Proferor Titular del Departamento de Fisiología (Fisiología Animal) de la Facultad de Veterinaria de la Universidad Complutense de Madrid, hacen constar:

Que la memoria presentada por el Licenciado en Veterinaria Pablo Bermejo Álvarez, con el título: "Sex ratio control before fertilization and preimplantational sexual dimorphism in bovine/Control de la proporción de sexos anterior a la fecundación y dimorfismo sexual preimplantacional en la especie bovina", ha sido realizada bajo nuestra dirección y que tras su revisión consideramos que tiene la debida calidad para su presentación y defensa.

Madrid, Febrero de 2010.

Fdo: D. Alfonso Gutiérrez Adán

Fdo: D. Pedro Luis Lorenzo González

Fdo: D. Dimitrios Rizos

EUROPEAN DOCTORATE MENTION

This thesis has been proposed for the European doctorate mention by virtue of the following European research stays and thesis reports:

Research stays:

- Lyons Research Farm, University College Dublin (Ireland). Dr. Pat Lonergan laboratory. 5 months in three stays during 2007-2008.
- School of Biosciences, University of Nottingham (United Kingdom). Dr. Keith Campbell laboratory. 4 months in 2009.

Thesis reports ("very good" grading):

- Isabelle Donnay, Unité des Sciences vétérinaires, Institut des Sciences de la Vie, Université Catholique de Louvain, Belgium.
- Giovanna Lazzari. AVANTEA, Laboratorio di Tecnologie della Riproduzione, Cremona, Italy.
- Pilar Coy Fuster. Facultad de Veterinaria, Universidad de Murcia, Spain.
- Rosa Ana Picazo González. Facultad de Veterinaria, Universidad Complutense de Madrid, Spain.

AGRADECIMIENTOS/ACKNOWLEDGES

Empiezo a escribir esta sección pensando en sus particularidades. A priori parece ser la menos relacionada con los resultados científicos que se presentan, pero quizás sea la que contiene los hechos más importantes para la obtención de los mismos. Es la única parte en la que se permite la subjetividad, y las afirmaciones no necesitan referencias o datos que las apoyen, puesto que tienen un destinatario concreto al que no le hacen falta pistas para saber de qué hablo. Además ni es evaluable ni va sufrir corrección alguna. Es por esto la más libre; de hecho no hay ni formato ni normas que la regulen, pero los usos y costumbres indican que suele ser corta. Sin embargo, pienso que caer en este costumbrismo sería injusto para demasiada gente. Ahí va:

A Alfonso, el principal responsable ideológico y logístico de que yo pueda presentar esta tesis. Si la calidad de un director de tesis se mide en la media de publicaciones por doctorando, creo que, sin exagerar, estás entre los mejores del mundo en este área. Gracias por confiar en mí, por introducirme en la biología molecular, por enseñarme técnicas y dejar que fuese desarrollando otras, por animarnos a ir a tantos congresos, por trabajar duro y funcionar a muchas más revoluciones que la mayoría, por tener las cosas listas para ayer, por inculcar ese "*yes, we can*" de la ciencia que incita a embarcarse en cualquier tipo de proyecto por complicado o irreal que pueda parecer, y por la libertad de elección y desarrollo de proyectos y de estancias.

A Pedro, porque algún día escribiremos un libro de peripecias y correspondencias. Gracias por acercarme a este mundo antes de licenciarme y por estar ahí en el momento más complicado de mi carrera científica. No es fácil encontrar por estos parajes gente tan honesta, buena persona y alejada del ego. Quizás por ello, estuviste en nuestra orla.

To Dimitrios Rizos, for teaching me everything about bovine IVF, for working together producing those thousands of embryos in Ireland, looking for cleavage rate at night or bleeding the cows over the Irish ice, for introducing me to all the Irish, and for being in charge of the international public relations of the lab.

To Pat Lonergan, who is morally another codirector of this thesis. Thank you very much for giving me total freedom and help to work in Lyons -without it, all this work could not have been possible- and for your essential input in the articles. It would have been very difficult to produce them without discussing with you before and after the experiments, and without your criticisms (sometimes you are the worse referee! hehe) and idiomatic changes.

To Detlef Rath, for providing sorted semen of an excellent quality, which was absolutely necessary for the experiments.

Al Ministerio de Educación y Ciencia, de Ciencia e Innovación y de Educación por concederme la beca-contrato FPU y las tres estancias breves que he disfrutado. Al INIA, centro de adscripción. A la asociación Precarios, por su lucha por las condiciones laborales de los investigadores, gracias a la cual al menos tengo derecho a paro. A los revisores de esta tesis, ya mencionados. To the reviewers of this thesis, already mentioned.

A mis compañeros del laboratorio de Alfonso, por comprender, sufrir o dejar estar (según se mire, jeje) al personaje que va y viene con embriones del mundo y deambula sobre máquinas de PCR. Para evitar luchas intestinas a la hora del desayuno, os nombraré en orden de proximidad a mi sitio. Raúl, compañero motero, PCRero y de frikadas, sabes que te quiero aunque siempre estemos dándonos la espalda, a ti te debo el protocolo de bisulfito del capítulo 4. Ricardo, gracias por animar la hora de la comida, ahora que cada vez somos menos los que nos quedamos. Alberto, nuevo miembro entusiasta del club de las qPCR, porque sin esas conversaciones del club de la comedia protagonizadas junto a Ricardo, las tardes no serían lo mismo, y porque cuando un cero molesta se quita “y apañao”. A Juande, que cambió de aires, por tantas y tantas horas pensando en arreglar esta casa… (y hasta aquí puedo leer, jeje). A Priscila. A Miriam, por estar siempre dispuesta a ayudar a todo aquel que entra “de nuevas” y por la compañía. A Eva, lider sindicalista de causas perdidas, “desfacedora de entuertos” y niña-melón, porque siempre animas con tu vitalidad, a veces pesimista y otras –menos- optimista ¿cómo andan esas células? A Sandra. A Alicia, que se fue a “Zarapower”, por tus locuras. A Miguel, compañero

ideológico y tío sensible que ve La 2, por saber sacar provecho del grupo control y por el apoyo informático que nos ofreces junto con Raúl.

A mis compañeras del laboratorio de Dimitrios. María, compañera de inicios bovinos, gracias por la convivencia en ca´la Tere y por tu ayuda en Irlanda. Celia, gracias por alegrar la hora de la comida y por soportar estoicamente todas nuestras bromas.

Al resto del personal del INIA. Particularmente a Miguel Ángel (así no se nota que repites), por todas las conversaciones -a veces muy profundas y otras subrealistas- y por esos momentos bohemios con o sin bucle. A Chica, Julio, Ernesto, Claudio, Raquel, Toñi, Bárbara, Elma y María.

A todos aquellos con los que hemos colaborado o han pasado por el INIA. A María y Rosa (laboratorio de Pedro), por todas las paradas técnicas en el camino de la cafetería al INIA. A Tere Zomeño e Irene Mondéjar (Universidad de Murcia); Piticli bonica, nunca te olvidaremos. To Daniella Sanna and Maria Dattena (Agris-Sardegna); Daniella, thank you for being so nice and funny and for your hospitality in Sassari. To Veerle van Hoeck, Ilse Goovaerts and Jo Leroy (University of Antwerp); Veerle and Ilse, thank you for your energy and enthusiasm (even being pregnant!). To Carmen Díez and Enrique Gómez (SERIDA), Roger Sturmey and Henry Leese (University of York), Daniella Bebbere (University of Sassari), and Ciara O´Meara and Manuel García (Pat´s laboratory) for collaborate with us.

To all of those I met during my stays in Lyons, my second home. To Theresa Clarke, the best ever landlord and the best brown bread at both banks of the Liffey, you should write a book with all the stories of the scientists you have provided accommodation… To Nora and Jerry Deegan, for the nice accommodation and for the Guinness at the GAA. A María Arias, gracias por toda la ayuda en la producción embriones, por compartir tanto SUG, por la compañía y por tantas discusiones sobre la vida becaria en las tierras de los O´Connors y de los Garcías. Tú si que eres grande. A mi 205, el campeón, porque sin él no hubiese podido hacer nada en Irlanda y casi nada en Nottingham. To Catherine Lawson, for “ovary” me so many times, and to Mary Wade, for her excellent technical work (you know how much I would like you

to be at INIA). To all the Kildare slaughterhouse staff, especially to Joe and Paul, for being so nice (I owe you much San Miguel beer). To Trudee Fair, for the interesting discussions about molecular biology and for allow me to make some trials in her laboratory. A Alejandro, Marina, Manu e Inés, por las cenas y pintas en castellano (y también por la pesca en el Ryewater y en el laguito de Maynooth, Alejandro). To Satoko (Sam), Salomon, Ciara, Francesca, Fionna, Pat Duffy, Serena and Abdullah, for the conversations, fun and pints.

To Xiuchun (Cindy) Tian (University of Connecticut), because although I arrived at a very bad moment, you were always really kind and helpful to me and finally I was able to learn the techniques and do some experiments. To Xiangzhong (Jerry) Yang, for his passion for science. To Chih-Jen (Lance) Lin, for teaching me SCNT in mouse, for your friendship and help and for lending me your bicycle! To Yong Tang, for his help to find accommodation and for that nice fishing trip and international dinner. To Fuliang Du, for teaching me his protocol for bovine SCNT. To Wei-Wen Lin for his advice in karyotyping and cell culture. To Tomokazu Amano, for his advice in fusion methods. To Mark Carter, Li-Ying Sung, Jooghoon Park, Kim Chul, Edwin and Bing Atabay, Misa Amano, Chin-Der Chen, Gosh and Yinghong Ma for their help and kindness.

To Keith Campbell (University of Nottingham), for giving me the opportunity to work in his laboratory and for the fruitful discussions. To Sandra Simpson for the kind accommodation. To Inchul Choi, for showing me the ovine SCNT. To Adel Moawad for his help in ovine IVF. To Pat Fisher, for her patience solving my problems. To Wein Chao Chen and Jie Zhu for their kindness. To Zoe Daniel, for the qPCR lab management. To Ramiro Alberio for the interesting discussions about SCs and Kevin Sinclair for that lift to the airport.

A la Facultad de Veterinaria en la que presento esta tesis, por los grandes años que pasamos, y a sus buenos profesores. A los compañeros de los departamentos de los que he sido interno: radiología, cirugía y anestesia de pequeños animales, microbiología y especialmente Fisiología Animal. To Fuller Bazer and Thomas Spencer (Texas A&M), for giving me the opportunity to learn in their laboratory. A PubMed y wikipedia, mis otras universidades. A mis compañeros de promoción, por

tantos buenos momentos: Coloma, Alberto, Cerce, Cris, Sandra, Deborah, María, Laura, Anahí, Inés, Ari, Nana, Cova, Raúl, Pilar, Jose Antonio, Juan, Chuko, Ramón, Pepe, Javi, Elenas, Macarena, Mayte, Bea, Sara, Eladio, Quiques, Luz, Almudena, Nicole, Miriam, Popi, Elisa, Clara, Ismael, Miguel, Isabeles, Mery... y especialmente a los otros tres magníficos, Babín, Torralbo y Chavi, componentes esenciales junto con sus respectivas –Mónica, Cata y Laura- de las tertulias de todólogos. Sirva de homenaje que el color de los separadores de capítulos sea el mismo que el de las entradas de las fiestas de veterinaria. A mis compañeros y profesores del colegio Gil-Díaz, barrio de Tetuán y del antiguo Instituto Jaime Vera, especialmente a Don Julio, Beatriz Luengo, María Jesús Peña y María Acebo. A los amigos de Aldea Real: Filimonas, rubias, Laura, María, Jesusín, Jesusón, Jose Ángel, Alberto, Davices… y especialmente a Miguel, compañero inseparable de pesca y juergas, y crack en sus ratos libres.

A mi familia, siguiendo el orden en el que lo pintaría un niño: A mi padre porque me inyectaste en el campo la pasión por los perros y otros "bichos" que ha desembocado en esto de la veterinaria. A mi madre, por todos tus esfuerzos para que superásemos todos nuestros problemas y por aguantar lo "graciosos" que somos, jeje. A mi hermano, autor de la portada de esta tesis, por ser incluso más amigo que hermano y por ayudarme tantas veces con programas gráficos. A mi hermana, porque desde que salí de casa te debo mucho tiempo, sobre todo llegando a las horas que llego a hacer visitas. A mi familia ampliada: tías, abuelos, primos… A mis perros, los que se fueron (Toby, Perla, Lucky, Cuco, Diana, Rayo y el gran Yaky), y los que están (Doly, Kyra y Atila), por crecer con ellos, socializarme y recordarme de qué va esto de la vida. Al campo y a los ríos, por enseñarme a no rendirme buscando liebres en los barbechos de enero y a distinguir entre casualidad y causalidad. A Madrid, donde regreso siempre fugitivo de la ciencia buscando el pulso de su "todo es ahora", por instruirme en el arte de la aceleración cuando el semáforo se está cerrando.

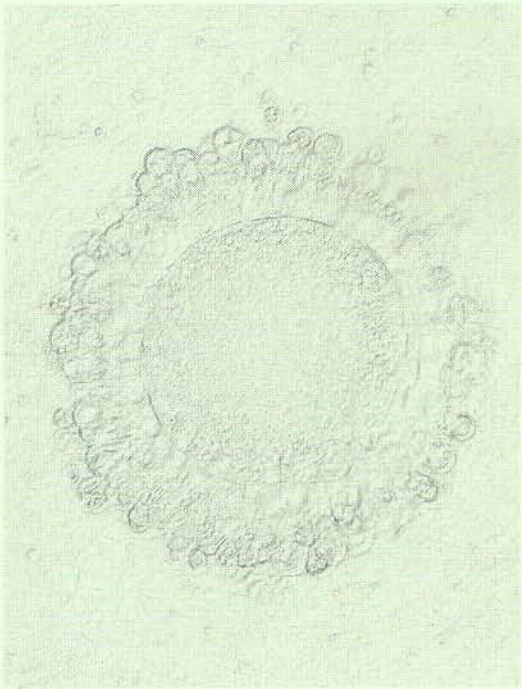
A Ana, mi incompatible favorita, porque eres la única persona que sabe casi todo lo que hay detrás de esta tesis y sus estancias. Por compartir buenos y malos momentos internos y externos, y estar siempre cerca, aunque físicamente pueda haber casi medio planeta de distancia. Por hacer siempre (bueno, casi, jeje) agradable la vuelta a la trinchera o campo base (según momentos). Por recargar ideales y analizar

situaciones, por los buenos días y las buenas noches, porque “tú sola sí te la sabes”, por saber qué plato tiene más, por las natillas, por vivir en pecado, por tantas aventuras en los “días sueltos” después de los congresos o estancias y por todo lo que con las prisas a veces olvido decirte.

A Ana.

INDEX/ÍNDICE

ABSTRACT/RESUMEN



ABSTRACT

One of the most recurrent mankind´s concerns along history is the gender of both its own offspring and that of the domestic animals. In animal production, sex is considered the most important genetic trait for animal production. Several studies have observed an interaction between maternal and paternal features or conditions and secondary sex ratios. Some maternal features such as her corporal condition, diet, glucose level, dominance status, testosterone level, stress, age, parity and litter size, and some female-linked parameters such as time of insemination, side of ovulation, exposure to contaminants and environmental temperature have been suggested to alter sex ratio. The complex relations between the different variables suggested to influence the sex of the offspring make these observations and their subsequent hypothesis rather speculative without a molecular or physiological basis. Mechanisms responsible for skewed sex ratios may occur at two distinct periods: prior to fertilization (preconceptional mechanisms) or after fertilization (postconceptional mechanisms). From an evolutionary point of view, mechanisms acting early (before or around conception) would be more advantageous than those acting later, as the last are more wasteful because they imply the loss of embryos or fetuses, with a subsequent loss of fertility. The general objective of the present thesis is to analyze possible preconceptional mechanisms for sex ratio control and to determine sex-related physiological, transcriptional and epigenetic differences in bovine preimplantation embryos.

Preconceptional mechanisms can be divided in two groups. The first one involves a putative spermatozoa selection by the oocyte which could alter the equality in fertilization chances between X- or Y-bearing spermatozoa. The second is based on putative intrinsic differences between X- or Y-bearing spermatozoa which could affect the equal chances for X- and Y-bearing spermatozoa to reach the fertilization site. In this thesis, the feasibility of the first will be discussed. A spermatozoa sex selection mechanism by the oocyte has been proposed to explain the relation between sex ratio and three different features of the oocyte: stage of maturation, testosterone concentration in the follicular fluid and the side of the ovary of origin. A putative spermatozoa sex selection affected by the maturation stage of the oocyte provides an explanation for the influence of the time of insemination in relation to the ovulation

upon sex ratio suggested for several species, including bovine. The effect of the maturation stage of the oocyte upon kinetics of first cleavage and embryo yield and sex ratio was examined in chapter 1. Maturation time affected kinetics of first cleavage and cleavage rate, but no has no effect on the sex ratio of the embryos produced.

Currently only three methods have proved to change the sex ratio of the offspring: the used of sex-sorted semen, sex determination by embryo biopsy and transgenic modifications. The first is the most widely used due to economic and practical reasons. Although accuracy of sexing by flow cytometry is around 90 % in most species, including cattle, its use is limited due to economic reasons. Among the different factors affecting the economic profit for the use of sorted semen, the reduced fertility of sexed sperm results in considerable deficits. Several factors have been proposed for the reduced fertility rates, including lower doses of spermatozoa used, damage to the spermatozoa caused by the sorting procedure, and a putative spermatozoa selection by the oocyte. If the oocyte can select preferentially X or Y-bearing spermatozoa, fertility rates using sex-sorted spermatozoa will remain low irrespective of the advance of the sorting techniques. The hypothetical spermatozoa selection by the oocyte was tested in chapter 2 by examining the differences in *in vitro* fertility between unsorted, sorted (X- or Y-sorted) and sorted/recombined (a mix of X- and Y-sorted) sperm. If the oocyte is able to select the sperm, a decrease in fertility would be obtained for both sorted groups (X- and Y-sorted), whereas fertility would improve for the sorted/recombined group. A decrease in fertility was found for all three groups fertilized with sorted semen compared with unsorted, which indicates that *in vitro* matured oocytes are not able to preferentially select sperm of one sex over the other, and thus the reduction in fertility is likely to be caused by the sperm damage produced by the sorting procedure.

To further investigate the effect of the sperm damage over the embryo development, the differences between sorted and unsorted semen in timing of first cleavage and blastocysts appearance, and in relative mRNA abundance of developmental-related genes were analyzed in chapter 3. The reduction in fertility following IVF with sex-sorted sperm is associated with a delay in the timing of the first cleavage, but no effect on the timing of blastocysts appearance was observed.

Moreover, the kinetics of first cleavage differs between sex-sorted sperm from 3 different bulls which suggest that susceptibility of sperm to the damage caused by the sorting procedure may differ between bulls, and that IVF could provide a valuable tool to test these differences in susceptibility, thereby allowing sire selection to obtain higher fertility rates following AI or further improvements in the sorting procedure. Although normal progeny is obtained by sorted sperm, it has been suggested that damaged spermatozoa may be able to fertilize an oocyte, resulting in low-quality embryos. A putative negative effect of the use of sorted semen on two embryo quality parameters (speed of development and gene expression) was analyzed. Neither timing of blastocysts appearance nor relative transcript abundance of genes related with apoptosis, detoxification of ultraviolet-induced oxygen radicals, pregnancy recognition, placenta formation and DNA methylation were affected by the use of sex-sorted sperm, suggesting that it does not affect embryo quality.

Postconceptional mechanisms affecting sex ratio may explain the sex ratio skews accompanied by a reduction in fertility, especially those observed under adverse or stressful conditions. It is known that preimplantation embryos may display sexual dimorphisms in terms of speed of development, cell count, survival in culture conditions and after vitrification, and gene expression, which occurs before gonadal differentiation and can only be attributable to their differences in sex chromosome dosage. Preimplantational sexual dimorphism provides a physiological basis for the different survival of male and female embryos under adverse conditions. However, this is not the sole implication for the preimplantation sexual dimorphism. The study of this phenomenon in early stages of development will help to understand phenomena such as sex chromosome transcriptional regulation, early X-chromosome inactivation, early sex determination, sexual chromosome evolution, X-linked diseases, and sex-specific long-term effects regarding to the embryonic origin of adult diseases.

Sex-related differences in speed of development and survival under *in vitro* conditions have been reported for several species. Sexual dimorphism in these parameters was analyzed in our culture conditions by two different approaches: embryo sexing after IVF with unsorted semen (chapter 1) or the use of sex-sorted semen (chapter 3). No differences in the timing of first cleavage and blastocysts

appearance and in embryo survival were found, indicating that adverse culture conditions may be the cause for the sexual dimorphism previously reported. Furthermore, a higher sex ratio in embryos produced by IVF with sorted or unsorted sperm was noted in chapters 1-3, suggesting a putative higher fertilization ability of the Y-bearing spermatozoa.

Epigenetic status, especially DNA methylation or histone methylation or acethylation levels, is the basis for transcriptional differences and thus, transcriptional sexual dimorphism should be originated by epigenetic events. However, during preimplantation development, X-chromosome inactivation remains the only sex-specific epigenetic difference reported. In chapter 4, sex-related differences in transcription of enzymes related with the epigenetic code, methylation status of genomic sequences, telomere length and mtDNA copy number were analyzed. A higher transcription for the epigenetic-related enzymes *DNMT3A*, *DNMT3B*, *HMT1* and *ILF3*, and a higher methylation level for the sequence *VNTR* were found in male embryos compared with females, suggesting a higher transcriptional level in female embryos. Shorter telomere length and higher mtDNA copy number were also found for male embryos compared with female.

Sex-related differences in transcription have been reported for both sex chromosome- and autosome-linked genes. However, with the exception of a microarray study carried out in the mouse model, all the studies have been based on a small number of genes. Global gene expression studies provide an estimation of the extent of the transcriptional sexual dimorphisms, which helps to understand phenomena such as sex chromosome transcriptional regulation, X-chromosome inactivation and sex ratio skews due to postconceptional mechanisms. A microarray study was performed on bovine male and female embryos produced with sex sorted spermatozoa in chapter 5. It was found that in the absence of hormonal differences, sex chromosomes impose an extensive transcriptional regulation upon autosomal genes, affecting to almost one third (2921) of the transcript detected. The array was validated with both *in vitro* and *in vivo* derived embryos produced with unsorted semen. Gene ontology analysis of the sexually dimorphic transcripts suggested a higher transcriptional level for female blastocysts and a more active protein metabolism, and an increased mitochondrial activity for males.

In eutherian mammals, X-chromosome inactivation (XCI) compensates the differences in X-chromosome dosage between male and female. However, some genes can escape from the X-chromosome inactivation process and be biallelically expressed leading to an upregulation of X-linked genes in females. This situation is especially common in preimplantation development, when XCI is a reversible and dynamic process. The microarray study results suggested that the bovine blastocyst, XCI is far from been accomplished, as most of the X-linked transcripts present in the array (88.5 %) were upregulated in females. However, most of them (70 %), exhibited a fold change lower than 1.66, which suggest partial XCI. A higher expression of X-linked genes in females is usually explained by a double allele expression, however, as males only contains the maternal inherited X-chromosome, a preferential paternal allele expression by an imprinting phenomenon may be involved. In chapter 5, possible imprinting mechanisms were tested by analyzing the differences in expression levels of eight X-linked genes between male, female and parthenogenetic embryos. Five out of the eight genes were found to be expressed preferentially by the paternal X-chromosome, which suggest that imprinting mechanisms may increase the transcriptional skew caused by double X-chromosome dosage. Finally, a novel gene (*YZRSR2*) homologue to an X-linked gene which encodes an essential splicing factor involved in network interactions during spliceosome assembly was found in the Y-chromosome.

RESUMEN

El género de la descendencia humana y animal ha sido una de las preocupaciones más recurrentes de la humanidad a lo largo de la historia. En producción animal, el sexo es considerado como el factor genético más importante. Varios estudios han observado una interacción entre características o condiciones maternas o paternas y la proporción de sexos secundaria. De este modo, se ha sugerido que algunas características maternas, como la condición corporal, la dieta, los niveles de glucosa, el estatus de dominancia, los niveles de testosterona, el estrés, la edad, el número de parto y el tamaño de camada, y algunos parámetros ligados a la hembra tales como el momento de inseminación, el lado de ovulación, la exposición a contaminantes y la temperatura ambiental, pueden alterar la proporción de sexos. Debido a la complejidad de las relaciones entre las diferentes variables que pueden influir en la proporción de sexos de la descendencia, estas observaciones y las hipótesis a las que dan lugar son bastante especulativas sin una base molecular o fisiológica. Los mecanismos responsables de un sesgo en la proporción de sexos pueden ocurrir en dos momentos distintos: antes de la fecundación (mecanismos preconcepcionales) o después de la fecundación (mecanismos postconcepcionales). Desde un punto de vista evolutivo, los mecanismos que actúan tempranamente (antes o alrededor de la concepción) serían más ventajosos que aquellos que aquellos que actúan posteriormente, ya que estos últimos provocan mayores pérdidas al implicar la pérdida de embriones o fetos y una correspondiente pérdida de fertilidad. El objetivo general de la presente tesis es analizar los posibles mecanismos preconceptuales de control de la proporción de sexos y determinar las diferencias fisiológicas, transcripcionales y epigenéticas entre embriones preimplantacionales bovinos de distinto sexo.

Los mecanismos preconcepcionales se pueden dividir en dos grupos. El primero implica una supuesta selección espermática llevada a cabo por el ovocito que puede alterar la igualdad de oportunidades de fecundación entre los espermatozoides X o Y. El segundo se basa en unas supuestas diferencias intrínsecas entre espermatozoides X o Y, que podrían alterar la equidad de probabilidades de alcanzar el lugar de la fecundación entre espermatozoides X o Y. En esta tesis, se discute la posibilidad del primer supuesto. Se ha propuesto que los mecanismos de selección

espermática del ovocito pueden explicar la relación entre la proporción de sexos y tres caracteríaticas del ovocito: estado de maduración, concentración de testosterona en el fluido folicular y lado del ovario de origen. Una supuesta selección espermática determinada por el estado de maduración del ovocito podría explicar la influencia del momento de inseminación en relación a la ovulación sobre la proporción de sexos propuesta en varias especies, incluyendo la bovina. En el capítulo 1 se examinó el efecto del estado de maduración del ovocito sobre la cinética de la primera división, la producción de embriones y la proporción de sexos. El tiempo de maduración afectó a la cinética de la primera división y a la tasa de división, pero no a la proporción de sexos de los embriones producidos.

En la actualidad, sólo existen tres métodos para alteran la proporción de sexos de la descendencia que hayan demostrado su eficacia: el uso de semen sexado, la determinación del sexo mediante biopsia embrionaria y la transgénesis. El primero es el que se emplea con más frecuencia debido a razones prácticas y económicas. Aunque la exactitud del sexaje mediante citometría de flujo está entorno al 90 % en la mayoría de las especies, incluyendo al ganado bovino, su uso es limitado por razones económicas. Entre los distintos factores que afectan al rédito económico obtenido al emplear semen sexado, la menor fertilidad del semen sexado conlleva considerables pérdidas. El descenso de la fertilidad se puede explicar por varios factores, como el uso de dosis bajas de espermatozoides, el daño espermático causado por la separación espermática y un supuesto mecanismo de selección espermática del ovocito. Si el ovocito puede seleccionar a los espermatozoides X o Y, las tasas de fertilidad obtenidas al usar semen sexado seguirán siendo bajas, a pesar del avance en las técnicas de separación espermática. La hipotética selección de espermatozoides del ovocito se probó en el capítulo 2, examinando las diferencias de fertilidad *in vitro* entre esperma sin sexar, sexado (X o Y) y una mezcla de sexados (X e Y). Si el ovocito es capaz de seleccionar al esperma, se obtendría un descenso en la fertilidad en los grupos fecundados con semen sexado (X o Y), que mejoraría con la mezcla de sexados. Se encontró un descenso en la fertilidad en los tres grupos fecundados con semen sexado comparados con el semen sin sexar, indicando que los ovocitos madurados *in vitro* no son capaces de seleccionar al esperma de un determinado sexo y que la reducción en la fertilidad parece ser debida al daño espermático producido por el procedimiento de separación espermática.

Para investigar con mayor profundidad el efecto del daño espermático sobre el desarrollo embrionario, en el capítulo 3 se analizaron las diferencias entre semen sexado y sin sexar en el momento de la primera división y desarrollo a blastocisto, y en la abundancia relativa de ARNm de genes relacionados con el desarrollo. La reducción de la fertilidad tras realizar FIV con semen sexado se asoció a un retraso en el momento de la primera división, sin verse afectado el tiempo de desarrollo a blastocisto. Además, la cinética de la primera división difirió entre esperma sexado procedente de tres toros diferentes, que puede deberse a diferencias entre toros en la susceptibilidad al daño espermático causado por el procedimiento de separación espermática. Así, la FIV puede ser una valiosa herramienta para probar estas diferencias en susceptibilidad, permitiendo la selección del semental para obtener altas tasas de fertilidad en IA, o para mejorar las técnicas de sexaje de semen. Aunque se ha obtenido descendencia normal tras el empleo de semen sexado, los espermatozoides dañados pueden fecundar al ovocito dando lugar a embriones de mala calidad. Por ello se analizó el posible efecto negativo del uso de semen sexado sobre dos parámetros de calidad embrionaria (velocidad de desarrollo y expresión génica). El empleo de semen sexado no afectó ni al momento de desarrollo a blastocisto ni a la abundancia relativa de transcritos de genes relacionados con apoptosis, detoxificación de radicales libres de oxígeno inducidos por ultravioletas, reconocimiento de la gestación, formación de la placenta y metilación de ADN, sugiriendo que no afecta a la calidad embrionaria.

Los mecanismos postconcepcionales que afectan a la proporción de sexos pueden explicar aquellos sesgos acompañados de una reducción en la fertilidad, especialmente aquellos observados bajo condiciones adversas o estresantes. Se sabe que los embriones preimplantacionales pueden mostrar un dimorfismo sexual en términos de velocidad de desarrollo, recuento celular, supervivencia en condiciones de cultivo y vitrificación, y expresión génica, que suceden antes de la diferenciación de las gónadas y sólo se pueden atribuir a las diferencias en la dosis de cromosomas sexuales. El dimorfismo sexual preimplantacional aporta una base fisiológica para las diferencias de supervivencia entre embriones macho y hembra bajo condiciones adversas. Sin embargo, esta no es la única implicación del dimorfismo sexual preimplantacional. El estudio de este fenómeno en estadios tempranos del desarrollo ayuda al conocimiento de fenómenos como la regulación de la transcripción de los

cromosomas sexuales, la inactivación temprana del cromosoma X, la determinación temprana del sexo, la evolución de los cromosomas sexuales, las enfermedades ligadas al cromosoma X, y los efectos a largo plazo específicos de sexo en el contexto del origen embrionario de las enfermedades del adulto.

Se han descrito diferencias entre sexos en velocidad de desarrollo y supervivencia bajo condiciones *in vitro* en varias especies. El dimorfismo sexual en estos parámetros se analizó en nuestras condiciones de cultivo mediante dos aproximaciones diferentes: sexaje de embriones producidos con semen sin sexar (capítulo 1) o el uso de semen sexado (capítulo 3). No se observaron diferencias en el momento de la primera división y desarrollo hasta blastocisto ni en la supervivencia embrionaria, sugiriendo que el dimorfismo sexual observado por otros estudios puede ser debido a condiciones de cultivo adversas. Además, la proporción de sexos de los embriones producidos por FIV con semen sexado o sin sexar fue superior a 1:1 (capítulos 1-3), lo que puede indicar una capacidad fecundante mayor de los espermatozoides Y.

El estatus epigenético, especialmente la metilación de ADN o los niveles de metilación o acetilación de histonas, es la base de las diferencias transcripcionales y, por ello, el dimorfismo sexual transcripcional debe tener su origen en sucesos epigenéticos. Sin embargo, la única diferencia epigenética ligada al sexo descrita durante el desarrollo preimplantacional es la inactivación del cromosoma X. En el capítulo 4, se analizaron las diferencias entre sexos en la transcripción de enzimas relacionadas con el código epigenético, el estatus de metilación de secuencias genómicas, la longitud telomérica y el número de copias de ADN mitocondrial. Se observó una mayor transcripción de las enzimas relacionadas con la epigenética *DNMT3A*, *DNMT3B*, *HMT1* y *ILF3*, y un nivel de metilación mayor en la secuencia *VNTR* en los embriones macho comparados con las hembras, lo que sugiere un mayor nivel de transcripción en los embriones hembra. Además se obtuvo un mayor contenido en ADN mitocondrial y una menor longitud telomérica en embriones macho con respecto a las hembras.

Se han descrito diferencias entre sexos a nivel transcripcional en genes ligados tanto a cromosomas sexuales como a autosomas. Sin embargo, con la excepción de un

estudio de *microarray* llevado a cabo en ratones, todos los estudio se basan en un pequeño número de genes. Los estudios de expresión génica global proporcionan una estimación de la extensión del dimorfismo sexual transcripcional que ayuda al entendimiento de fenómenos como la regulación de la transcripción de los cromosomas sexuales, la inactivación del cromosoma X y los sesgos de la proporción de sexos debidos a mecanismos postconcepcionales. En el capítulo 5, se llevó a cabo un estudio de *microarray* en embriones bovinos macho y hembra producidos con semen sexado, en el que se observó que en la ausencia de diferencias hormonales, los cromosomas sexuales imponen una extensa regulación transcripcional sobre los genes autosómicos, que afecta a casi una tercera parte (2921) de los transcritos detectados. El *array* se validó con embriones derivados *in vitro* e *in vivo* producidos con semen sin sexar. El análisis de ontología génica de los transcritos que mostraron dimorfismo sexual sugirió un nivel de transcripción más alto en los blastocistos hembras y un mayor metabolismo proteico y actividad mitocondrial en los machos.

En mamíferos euterios, la inactivación del cromosoma X compensa las diferencias en la dosis de cromosomas X entre machos y hembras. Sin embargo, algunos genes pueden escapar del proceso de inactivación del cromosoma X y ser expresados de forma bialélica dando lugar a una sobreexpresión de los genes del cromosoma X en hembras. Esta situación es especialmente común durante el desarrollo preimplantacional, cuando la inactivación del X es un proceso dinámico y reversible. Los resultados del estudio de *microarray* sugieren que en el blastocisto bovino, la inactivación del cromosoma X no está completa, ya que la mayoría de los genes ligados al X presentes en el *array* (88.5 %) están sobreexpresados en hembras. Pese a ello, la mayoría (70 %) mostraron un cambio de expresión inferior a 1,66, sugiriendo una inactivación parcial del X. La sobreexpresión de genes ligados al X en hembras se suele explicar como consecuencia de la expresión de ambos alelos, pero también puede influir una expresión preferente del alelo paterno debido a un fenómeno de impronta genómica parental, dado que los machos sólo contienen el cromosoma X materno. En el capítulo 5, se buscó la existencia de mecanismos de impronta genómica parental analizando las diferencias en el nivel de expresión de 8 genes del cromosoma X entre embriones macho, hembra o partenotes. Cinco de los ocho genes se expresaron preferencialmente mediante el cromosoma X paterno, sugiriendo que los mecanismos de impronta genómica parental pueden incrementar el

sesgo transcripcional causado por la doble dosis de cromosomas X. Finalmente, se descubrió un nuevo gen (*YZRSR2*) presente en el cromosoma Y, homólogo a un gen ligado al cromosoma X que codifica para un factor esencial para el ensamblado alternativo de transcritos implicado en la red de interacciones del ensamblado del espliceosoma.

INTRODUCTION/INTRODUCCIÓN

INTRODUCTION

Sex is the individual feature which has the biggest impact on the mammalian phenotype. One of the most recurrent mankind´s concerns along history is the gender of both its own offspring and that of the domestic animals. As a sample of this concern, the first thing our parents are usually told about us is what sex we are, gender is the only genetic feature indicated in the identity documents from diverse countries, and in most languages, substantives has a gender. Therefore, it is not surprising that the allocation of sex in mammalian offspring had been object of study from ancient times [1].

Different cultures have believed in diverse methods to control the sex of the offspring. Greek philosophers firstly proposed sidedness as the origin of sex determination, based on the association of the right side with the goodness, the sun, the hot and the man and the left side with the evil, the moon, the cold and the woman. Parmemides (ca. 515 B.C.) proposed that the sex of the embryo was determined by the side of the womb in which it developed, whereas Anaxagoras (500 to 428 B.C.) exposed that right testicle was responsible for the male conception, and left for female, proposing the ligation of the left testicle to conceive sons [1]. The idea was maintained in "*Corpus hippocraticum*" (centuries 5^{th} and 4^{th} B.C.), and Hindu Tantric texts (7^{th} to 17^{th} century) taught a variation, declaring that if at the moment of orgasm the "solar breath" taken via the right nostril, dominates in man and the "lunar breath", taken via the left nostril, dominates in woman, and conception occurs, the child will be male, whereas the opposite was suppose to yield females [2]. French noblemen were still advised for removal of the left testicle to conceive sons [3], and Millot, the obstetrician of Queen Marie Antoinette of France (1820), wrote "*it is the last movement of the woman that determines the sex of the child: it is the side on which she lies at ejaculation time that drives to sex of the child: always a boy when she is on the right side and always a girl on the left side*" (cited in [4]). Aristotle (384-322 B.C.) criticized both Parmemides´ and Anaxagoras´ theories citing evidence that embryos of both sexes can be found in the same side of the uterus and that men with a single testicle father children of both sexes [1] and proposed that the likelihood of having a male correlated directly to the vigor with which one copulated [2]. Empedocles (494-434 B.C.) claimed that the timing of intercourse could affect the sex

ratio because of the differences in the "amount of hot", and the idea has been maintained until nowadays in the Shettles method [5].

In Hebraic culture, Talmud (5th century BC), based on *Leviticus 12:2*, claims that an orgasm by the woman before the man selected for male offspring [4]. Talmud also suggested that placing the marriage bed in a north-south direction favored the conception of boys [4]. Asiatic cultures believed in an influence of the astronomy on sex ratio. In South Korea it is traditionally consider that the year of the Horse bears inauspicious implications for the birth of daughters [6]. Chinese gender selection chart takes the woman´s lunar age and matches it with the lunar month of conception to predict the sex of the offspring. Indian Ayurvedic texts by Sushruta (5th century B.C.) and Charaka (2nd century B.C.) suggested the use of different herbs combined with astrological events at 2 months of gestation to increase the probability to engender a son [7]. The notion has persisted throughout the time and sex selection drugs based on plants such as Shivalingi (*Bryonia laciniosa*) and Majuphal (*Quercus infectoria*) are currently used in rural India [7]. The Spaniard Huarte San Juán joined diverse folk methods in his book "*Examen de ingenios para las ciencias*" (1575) which includes several advices for the man, regarding to diet, exercise, and time and position of intercourse. Some symbolic methods, such as the Germanic folk which suggested that the man should carry an axe to the bed in order to conceive sons [4], were used in Czechoslovakia and Hungary, Palau Islands, Yugoslavia and Italy [2].

At the end of the 19th century, L Cuénot said about the methods to control the sex of the offspring in his book "*Fin the siècle*": "*It is surely humiliating to state that as regards man and others mammals, no advance has been made since the time of the predecessors of Aristotle*" (cited in [1]). A mayor breakthrough was the discovery that sex in mammals was determined by the sex chromosomes [8], which challenged all postconceptional theories. In the 20th century, Shettles method, described in his book "*How to choose the sex of your baby"* [5], is probably the most known natural strategy. It is based on putative difference in motility and survival between Y- and X-bearing spermatozoa, and it claims that assuming certain sexual positions, the timing of intercourse relative to the menstrual cycle determines the sex of the baby. The accuracy of the method has been seriously criticized by the scientific community [9], and currently only three methods have proved to change the sex ratio of the offspring:

the used of sex-sorted semen, sex determination by embryo biopsy and transgenic modifications [10]. Despite this fact, an internet search for alternative methods will show diverse advice and products to obtain offspring of a predetermined sex including different diet, caffeine or chocolate ingestion, “specially balanced nutraceuticals”, testicular temperature, vaginal pH, orgasm, sexual positions, position of the moon relative to astrological houses, lunar phase, specific dates… and Shettles book was published in its 6th edition in 2006.

Sex is considered the most important genetic trait for animal production [11], as it exert a great influence in growth rates, milk production and susceptibility to diseases. As a genetic trait, sex is a special case that can not be manipulated by genetic selection, but only by sex predetermination methods. Some studies have determined the economical benefits of a sex predetermination system in animal production. In dairy cow, only female cattle are productive, as male dairy cattle display poor growth performance for beef production. It has been estimated that a 30 % increase in milk production efficiency could be obtained if female offspring could be selected at the time of insemination [12]. Similarly, in beef cattle, a 20 % increase in the gross margin was calculated for an increase from 50 % to 90 % male [13].

Sex ratio is the proportion of male offspring relative to female offspring. Spermatogenesis transforms the diploid spermatogonia into haploid spermatozoa, producing an equal amount of X and Y-bearing spermatozoa. Assuming that X and Y-bearing spermatozoa displayed equal survival/transport in the female genital tract and equal fertilization ability, and that both sexes are subjected to an equal mortality rate, sex ratio should be 1:1. Sex ratio can be measured at different time points: Primary sex ratio is the male-to-female sex ratio at the time of conception, secondary sex ratio is measured at birth, tertiary sex ratio is determined at puberty and quaternary sex ratio is observed at the end of the reproductive age. Under normal circumstances, all mammalian species display a secondary sex ratio roughly similar to 1:1. However, several studies have observed an interaction between maternal and paternal features or conditions and secondary sex ratios. The term “increased” or “high” sex ratio refers to a significant variation of the theoretical sex ratio in favors of males, whereas the opposite (“decreased” or “low”) can be said for a female-biased sex ratio. A variable

which shows “a positive correlation with sex ratio” increases the sex ratio, whereas “negatively correlated” variable yields to a low sex ratio.

Female-based sex ratio skews

It has been suggested that as mammalian female´s contribution to nurturing her young is more costly than male´s, both pre- and post-natally, it would make intuitive sense for her, from an evolutionary point of view, to have at least some input into sex allocation [14]. Some maternal features such as her corporal condition, diet, glucose level, dominance status, testosterone level, stress, age, parity and litter size, and some female-linked parameters such as time of insemination, side of ovulation, exposure to contaminants and environmental temperature have been suggested to alter sex ratio.

Among these factors, the nutritional condition of the mother has drawn a special attention. A theory specially applicable to polygonous species and known as Trivers and Willard hypothesis, suggests that as maternal condition declines, the adult female tends to produce a lower ratio of males to females, because a male in good condition at the end of the period of parental investment is expected to outreproduce a sister in similar condition, and she is expected to outreproduce him if both are in poor condition [15]. Furthermore, it seems logical that mothers in poor condition should produce female-biased offspring, as the female requires higher resources to grow a male fetus than a female. There are several evidences which links a high plain of maternal nutrition with an increased sex ratio (reviewed in [16]). Retrospective studies have associated male-biased sex ratio with good maternal condition in humpback whale (*Megaptera noraeangliae*) [17], red deer (*Cervus elaphus)* [18, 19], reindeer (*Rangifer tarandus*) [20], horse [21], bovine [22] and human [23], whereas female biased offspring has been observed for low maternal weight or under unfavorable circumstances in horse [21], rat [24], and in human in first [25, 26] or second [26] pregnancy. In contrast, no effect of maternal body condition was found for bighorn sheep (*Ovis canadensis*) [27]. Change in body condition, rather than body condition itself has been proposed as the sex ratio skew causing mechanism via glucose concentration. In this perspective, mares gaining condition showed a extreme male-biased offspring, whereas the opposite was true for those loosing condition [28].

Has nutritional condition an effect upon sex ratio, it might be possible a nutritional control of the offspring sex ratio. Consistently with a male-biased offspring for mothers in good condition, high calorie diets were found to increase sex ratio in opossum (*Didelphis marsupialis*) [29] and fallow deer (*Dama dama*) [30]. Similarly, in dairy cows, but not in heifers, a high plane of nutrition was reported to lead to a male biased offspring compared with a poorer diet [31], and in human, high caloric availability has been positively correlated with sex ratio [32]. In the same trend, high maternal glucose levels during fertilization have been found to be correlated with high sex ratios in field vole (*Microtus agrestis*) [33], and diabetic mouse produces male-biased offspring [34], although in human it has been reported the opposite situation [35]. On the other hand, poor maternal condition is associated with poor diet, which should lead to female biased offspring. Diet restriction during pregnancy was claimed to decrease the sex ratio in mice [36]. In the same specie, low fat diet was found to produce a 1:3 sex ratio [37], and an intermittently food deprivation for 1 week before mating produced a female biased offspring [38]. Food restriction also favored the production of female offspring in rat [39]. In contrast, in white-tailed deer (*Odocoileus virginianus*), poor diet was associated to male-biased offspring [40]. Although most of the studies have been focused on the caloric content of the diet, the effect may be caused by the level of some specific components. Thus, a diet isocaloric to the control but with high fat content increased the sex ratio in mice [41]. A high-fat diet can result in higher levels of circulating glucose, thereby supporting the hypothesis that glucose may be contributing to sex allocation [42]. In the same trend, a diet isocaloric to the control with increased content of rumen-protected polyunsaturated fatty acids (PUFA) increased the sex ratio of day 13 ovine conceptuses [43] without increase glucose levels, so the authors proposed two possible nutritional effects other than a nutritional effect on the embryo: the diet may delay ovulation and thus increase the sex ratio –as it will be discussed below-, or there may be a preferential recruitment of Y-bearing spermatozoa due to the accumulation of n-6 (omega 6) PUFA in the oocyte [43]. In contrast, in mice a diet with increased omega 6 PUFA content skewed the sex ratio in favor of females, whereas no effect was observed for a diet with high omega 3 content [44]. Other diet components have been also reported to alter sex ratio. Mineral compound were suggested to affect sex ratio in early studies in bovine (cited in [4]). An inverse relation between sodium dietary content and sex ratio was noted in rat [45], and later it was reported in the

same specie that dietary calcium and magnesium supplementation combined with sodium and potassium reduction decreases sex ratio, probably due to a decrease glycerylphosphorylcholine diesterase activity [39].

Another factor suggested to affect sex ratio is maternal dominance, which has been also linked with maternal condition and diet. Dominant mothers are supposed to be in a better condition than their subordinates, and therefore both factors are usually associated leading to increased sex ratio in dominant mothers [18, 19], although "good condition" is not always a sign of maternal dominance [46]. Two opposite hypothesis have been proposed. Firstly, one hypothesis [47] concerning matrilineal primate species states that a dominant mother would leave more descendants through a daughter rather than a son if the daughter inherited her high rank, whereas a low ranking mother of such specie would leave more descendants through a son, for he is likely to emigrate at puberty and not necessarily inherit his mother´s low rank. This situation has been reported in captive rhesus monkeys (*Macaca mulatta*) [47] and bonnet macaque (*Macaca radiata*) [48]. On the other hand, the second theory, which is more widely supported and fits with maternal condition, establishes that a dominant female tends to produce more male offspring. Several reports support the second theory, as dominant female mammals has been found to produce more male offspring in both wild and captive populations of red deer (*Cervus elaphus*) [18], swine [49], Saharan arrui (*Ammotragus lervia sahariensis*) [50], barbary macaque (*Macaca sylvanus*) [51] and long-tailed macaque (*Macaca fascicularis*) [52], whereas bottom-ranking female were observed to produce more female pups in mouse [53] and barbary macaque (*Macaca sylvanus*) [51]. A similar situation has been observed in human [54], and a more complex link between maternal age and dominance was found for baboon (*Papio cynocephalus*): subordinate females give birth to more sons when they were young and dominant females had more sons when they were old [55]. Dominance is related to maternal testosterone levels (reviewed in [56]). Consistently, high maternal testosterone levels have been associated with increased sex ratio in bovine [56] and field vole (*Microtus agrestis*) [33], and two parameters influenced by testosterone/estradiol concentration -i.e. waist-to-hip ratio and orgasm ease- were positively correlated with sex ratio in human [57]. Contrary to both theories, two meta-analyses found the maternal dominance effect upon offspring sex ratio particularly inconsistent across the literature [42, 58], and some studies carried out in

yellow baboon (*Papio cynocephalus*) [59] and rhesus monkey (*Macaca mulatta*) [60] failed to establish a relation. Thus, it has been suggested that the previously observed effect could be the product of stochastic variation in small samples [58].

One of the factors that has been widely associated with altered sex ratio is the time of mating or insemination in relation to ovulation (reviewed in [61]). The notion was firstly mentioned by Empedocles in the fifth century B.C., and has persisted until nowadays by the Shettles method [5]. Some studies associate early insemination with low sex ratio and late mating with increased sex ratio in hamster [62], white-tailed deer (*Odocoileus virginianus*) [63], ovine [64] and bovine [65, 66]. In contrast, a lower sex ratio was observed for late mating in mouse [67], bovine [68] and human [69, 70]. Other studies reported an inverted-U shape kinetics, with low sex ratios in early and late inseminations in bovine [68] and in human following artificial insemination [71]. The U-shape has been proposed for rat [72] and human [73]: females are formed disproportionately often in the middle of the fertile window and males at either end of it. This tendency was also observed following natural insemination [71]. Nevertheless, some studies have found no relation between time of insemination in relation to ovulation and sex ratio in rabbit [74], swine [75], bovine [61, 76, 77] and human [9].

Another factor that has been though to influence sex ratio from ancient times is the side in which fertilization-gestation takes place. The idea was firstly offered by Parmemides in the 6th century B.C. which associated right side with male offspring and left side with female as a sex determination mechanism. Currently, the idea persists, although the modern explanation is a putative oocyte selection of Y- or X-bearing spermatozoa. In agreement with Parmemides, the sex ratio of calves gestated in the right horn has been reported to be significantly higher than the sex ratio of left-horn-gestated calves [78, 79]. Consistently, in Mongolian gerbil (*Meriones unguiculatus*) significantly more males were obtained from the right transplanted ovary (either on right or left horn) than from the left transplanted ovary [80]. However the opposite situation (higher sex ratio in the left horn) has been found in rabbit [81] and mouse [82], other studies have reported no differences in mouse [83], rat [84], and swine [85] -although in the last specie uterine migration usually occurs-, and the idea that the ovary of origin may influence the sex of the offspring has been

ridiculized [86]. In a similar line of embryo location and sex ratio, no sex ratio skew was found between three intrauterine segments (ovarian, middle, and cervical) in mouse [87].

Stress has been often linked with low sex ratios. Stressed mothers produced female-biased litters in rat [88] and hamster [89]. These observations have been supported by mechanistic studies which have found a decreased sex ratio in rats treated with ACTH [90] or a recovery of the normal sex ratio in stressed hamsters treated with dexamethasone [91]. In human there are also evidences towards a negative correlation between sex ratio and stress. For instance, a decrease in sex ratio has been found after acute stress caused by severe life events [92], natural disasters such as London smog of 1952 and Brisbane flood of 1965 [93] and Kobe earthquake of 1995 [94], or by a short war [95] or terrorist attack [96], and the amount of daily doses of antidepressants and anxiolytics in Sweden was inversely related to sex ratio [97]. However, in field vole (*Microtus agrestis*), no association was found between sex ratio and corticosterone levels [33] and chronic stress related to local resource competition has been associated with raised sex ratios in marsupials [98] and primates [99]. The same situation was observed following war in humans [100, 101]. As a possible explanation for these findings, sex allocation theory predicts that in a population with biased operational sex ratio (OSR), parents will increase their fitness by adjusting the sex ratio of the progeny towards the rarer sex [102]. In agreement with this theory, it was reported that more sons were produced when males were rarer than females in the majority of the 21 parishes analyzed in the pre-industrial Findland, where 90 % of the marriages were contracted within each parish [103]. In wolf pups it has been reported a skew in favor of males when the population density is high and no skew or female-biased when the population density is low [104], and similar findings have been reported for lesser mouse lemur (*Microcebus murinus)* [105] and wild yellow baboons (*Papio cynocephalus*) [59], and for black rhinoceroses (*Diceros bicornis*) in captivity [106].

Maternal age, parity and litter size have been also reported to exert an influence upon sex ratio. Maternal age has been negatively correlated with fetal sex ratios in white-tailed deer (*Odocoileus virginianus*), with the does older than 4.5 years conceiving more females and those younger than 3.5 conceiving more males [107]. A

curvilinear relation between sex ratio and maternal age was found in hamster mated once: females mated at 100 days of age obtained more males than those mated at 55 days, but less than those mated firstly after 325 days, which was explained as a consequence of the higher susceptibility to stressors in male embryos compared with their female counterparts [108]. The opposite curvilinear relation was found in ovine [50], with a female bias for young and old sheep and male bias for middle age sheep. Similarly, a positive correlation between maternal age and sex ratio was found in field voles (*Microtus agrestis*) [33] and bovine [109]. In humans, a negative correlation between sex ratio and age was found in Hungary [110], USA [111], and Spain [112]. However, other study found no differences in rhesus monkey [60]. Regarding to parity, a multivariate analysis applied to 1.4 millions of births found a weak negative correlation between birth order and sex ratio in USA [113] and similar findings were found in England and Wales population [114]. In hamster, an increase in the sex ratio was found up to the third parturition, when it started to decrease [108]. However, no effect was found in bovine [76, 115] and rhesus monkey [60]. Finally, in agreement with the effect of body condition, a negative correlation between sex ratio and litter size was found in guinea pig [116] and wild boar [117], which is likely to be cause by a higher uterine mortality for males. However, no effect was found in swine [75] and the opposite effect was observed in field vole (*Microtus agrestis*) [33].

Finally, secondary sex ratio has been proposed as a sentinel for environmental change [118], as exposure to contaminants has been linked with sex ratio skews. Low sex ratios have been associated with high contaminant exposure in human. The sex ratio in Italian major cities was reported to be lower compared with the rest of the country, whereas it was higher among the stillbirths. The authors hypothesized that a higher mortality rate for male fetuses in major cities due to exposure to hazardous environmental conditions may cause the skew [119]. Similarly, sex ratio was low in areas at risk for high pollution from incinerators in Scotland [120] and after the exposure to high levels of dioxins, the population of Seveso (Italy) experienced a decrease in both fertility and sex ratio [121]. Similarly, maternal (but not paternal) ingestion of polychlorinated biphenyls was found to reduce sex ratio in Great Lakes region population [122]. Tobacco smoking habits also were suggested to increase [123], to reduce [124] or to do not have any effect on it [125, 126]. Genetic background, in particular *ACP1* allele, has been suggested to be responsible for the

discrepancies among studies based on smoking habits [127]. Caffeine has been reported to slightly reduce sex ratio (61 % female) in Chinese hamsters (*Cricetulus griseus*) [128], and a skew of the sex ratio toward females was found in humans exposed to high voltage electricity in Bagdag [129]. Low sex ratios have also been associated to adverse medical conditions (reviewed in [130]), such as non-Hodgkin lymphoma [131], hepatitis [132], the use of fertility drugs such as clomiphene [132] congenital adrenal hyperplasia [133] and *Cytomegalovirus* infection [134, 135]; although increased sex ratio was observed for hepatitis B [136], breast cancer [137] and after *Toxoplasma gondii* infection in human [138] and mouse [139]. Weather has also been associated with sex ratio. Air temperature and humidity were positively linked with sex ratio in bovine [115] and male calves are more likely to be born in the warmer months of the year [109]. Similar findings have been reported for human, with more male births reported in warmer years in Finland [140], and an influence of the month of birth of the mother and the son was suggested for French Canadian [141] and Modena [142] population.

Table 1: Correlations between different maternal features and sex ratio.

Maternal feature	Positive correlation with sex ratio	No effect	Negative correlation with sex ratio
Body condition	Males in good condition: Humpback whale [17], red deer [18, 19], reindeer [20], horse [21], bovine [22] and human [23]. Females in bad condition: Horse [21], rat [24], and human [25, 26]. Males when body condition increases: Horse [28].	Bighorn sheep [27].	
Diet caloric content	Males in high calorie diet: Opossum [29], fallow deer [30], diary cows [31], and human [32]. Females in low calorie diet: Mouse [36-38] and rat [39].	Diary heifers [31].	Males in restricted diet: white-tailed deer [40].
Glucose level	Field vole [33] and mouse [34].		Human [35].
High-fat isocaloric diet	Total fat: Mouse [41]. PUFA: Ovine [43].		n-6 PUFA: Mouse [44].
Na^+ content in diet			Rat [39, 45]
Dominance	Males in dominant mothers: Red deer [18], swine [49], Saharan arrui [50], barbary macaque [51], long-tailed macaque [52], old baboons [55] and human [54]. Females in subordinate mothers: mouse [53] and barbary macaque [51].	Baboon [59] and rhesus monkey [60].	Rhesus monkey [47], bonnet macaque [48] and young baboons [55].
Testosterone level	Field vole [33], bovine [56] and human [57].		
Time of insemination	Hamster [62], white-tailed deer [63], ovine [64] and bovine [65, 66].	Rabbit [74], swine [75], bovine [61, 76, 77] and human [9].	Mouse [67], bovine [68] and human [69, 70].
Time of insemination (U-shape)	Inverted U: bovine [68] and human [71].		U: rat [72] and human [71, 73].
Side of ovulation/gestation	Males in right: Mongolian gerbil [80] and bovine [78, 79].	Mouse [83], rat [84] and swine [85]	Males in left: Rabbit [81] and mouse [82]
Stress	Chronic: Marsupials [98], primates [99] and human [100, 101].	Corticosterone level: Field vole [33].	Acute: Rat [88, 90], hamster [89, 91] and human [92-97].
Age	Field vole [33] and bovine [109]. Inverted U-shape: Ovine [50].	Rhesus monkey [60].	White-tailed deer [107] and human [110-112]. U-shape: Hamster [108].
Parity	Inverted U-shape: hamster [108].	Bovine [76, 115] and rhesus monkey [60].	Human [113, 114].
Litter size	Field vole [33].	Swine [75].	Guinea pig [116] and wild boar [117].
Chemical or physical environmental conditions	Hot weather: Bovine [109, 115] and human [140].		Contaminants: Human [119-122]. Caffeine: Chinese hamster [128]. High voltage: Human [129].
Tobacco (human)	[123]	[125, 126]	[124]
Adverse medical conditions (human)	Hepatitis B [136], breast cancer [137] and after *Toxoplasma gondii* infection in human [138] and mouse [139].		Non-Hodgkin lymphoma [131], hepatitis [132], clomiphene treatment [132], congenital adrenal hyperplasia [133] and *Cytomegalovirus* infection [134, 135].

Male-based sex ratio skews

Most of the studies of the secondary sex ratio are focused on a putative maternal effect, but some articles have reported a paternal effect upon sex ratio. Apart from X-linked diseases which result in increased sex ratios due to female mortality, it is difficult to provide a biological mechanism for a paternal-based sex ratio skew, as male produce an equal amount of X- and Y-bearing spermatozoa [143, 144]. Thus, putative paternal effects on human secondary sex ratio have been suggested to be the result of second-order effects due to assortative mating [46].

In human, a tendency towards a negative correlation between paternal age and sex ratio has been suggested [113, 114, 145]. However, the percentage of Y-bearing spermatozoa was not affected by age [146] and therefore an interaction between maternal age and parity and paternal age may be the cause for the differences. It has been also proposed that rather than the age of either parent, the difference in age between parents may be predictive for the sex of the first children: parents with a greater difference between their ages gave birth to an excess of boys, and the opposite situation was found for small age differences [147].

A positive relation between male fertility and the proportion of male offspring has been found in red deer [148]. The study avoided the interaction with female factors by artificial insemination, and thus, the authors hypothesized that the effect could be caused either by a different proportion of X- and Y-bearing spermatozoa in the ejaculate or by a putative fertility advantage of the Y-bearing spermatozoa in relation to X-bearing when produced by more-fertile males, whereas the opposite may occur among less-fertile males. The first hypothesis is supported by the finding of a variation in sex ratio between ejaculates within sire in bovine [149], although these results have not been repeated. The second can be explained by the presence of Y-chromosome deletions in the low fertility males, as it has been found in the mouse model that Y-chromosome deletions produce Y-bearing spermatozoa with morphological abnormalities that are less efficient at fertilization, resulting in lower sex ratios [150]. Consistently with a link between fertility and sex ratio, men with adverse medical conditions such as testicular cancer [151, 152], or exposed to adverse chemicals such as dioxin [121], dithiothreitol [153], dibromochloropropane [154],

fungicides [155], boron [156], aluminum residues [157], or tobacco [124, 158] father a female-biased offspring which has been correlated with low paternal testosterone [159], whereas men treated with methyltestosterone therapy sired more boys than girls (45:17) [160]. In a similar trend, experimental pre-mating administration of dioxin to the males decreased the sex ratio in mouse [161] and guinea pig [162]. However, slight reductions in fertility has been found to do not alter sex ratio in human [163]. Semen collection frequency has been also suggested to exert an effect upon sex ratio in bovine [164]. Finally, in a similar line than paternal effects, artificial insemination has been suggested to increase the probability of a male calf in both dairy and beef cattle, although an increase of just 1 % was notice [109]. The same study also suggested that beef breed sires skew the sex ratio towards males [109].

Biological mechanisms for parental influences on sex ratio

Most of the sex ratio skew observations cited above are based on retrospective studies, which lack a biological mechanism support to be fully reliable. The complex relations between the different variables suggested to influence the sex of the offspring (i.e. maternal condition, dominance, glucose, testosterone, time of insemination…) make the observations and their subsequent hypothesis rather speculative without a molecular or physiological basis. Indeed, it has been suggested that some of the sex ratio alterations reported are not real, but just caused by hazard [86]. Due to its importance and easy recording, sex ratio is a common parameter to be analyzed, which is rarely overlooked in any kind of analysis of an animal population. Thus, thousands of sex ratio analyses are performed each year in very diverse studies involving different species and parameters. Within such a number of observations, some of them would display significant differences, and these would be the only ones to be published. For these reasons, mechanistic studies together with meta-analyses and physiological markers are necessary to find the real meaning of different observations.

When consistency and cohesiveness of the different articles published have been tested by meta-analyses, they usually concludes that the observed effect can be the product of stochastic variation in small samples [58]. However, a review restricted to ungulates found consistent support for a relationship between maternal dominance,

maternal condition and offspring sex ratio and that maternal condition measures taken around conception provide the most consistent support for the Trivers and Willard hypothesis [165]. Consistently, another meta-analysis based on mammalian studies, excluding human, determined that sex ratio adjustment occurs at or near implantation [42]. Furthermore, in domestic species, the beginning of lactation, the most energetic demanding period, coincides in time with of conception, and thus this period is the most reliable one to establish a relation between body condition and mother´s future ability to invest energy.

Two physiological markers have been suggested to explain the discrepancies between studies. First, maternal glucose concentration around conception has been proposed as the link among different results [42], as it is high for mothers in good condition, but also increases under social stress, is high in diabetes and change in adverse medical conditions, varies through the cycle, and is related to LH [166, 167]. Secondly, maternal testosterone levels have been suggested as a plausible explanation for the contradictory findings on maternal condition, dominance range and stress [14] -as chronic stress affects testosterone levels by decreasing in male and increasing in female-, and for the sex ratio skews caused by different adverse medical conditions and contaminants [168].

Mechanisms responsible for skewed sex ratios may occur at two distinct periods: prior to fertilization or after fertilization via selective loss of embryos/fetuses of one sex relative to the other. In most descriptive studies, it is difficult to establish whether the atypical sex ratios had their origin at conception or during pregnancy or both, since the same environmental effects pertained both before and after conception [14]. However, from an evolutionary point of view, mechanisms acting early (before or around conception) would be more advantageous than those acting later, as the last are more wasteful because they imply the loss of embryos or fetuses, with a subsequent loss of fertility.

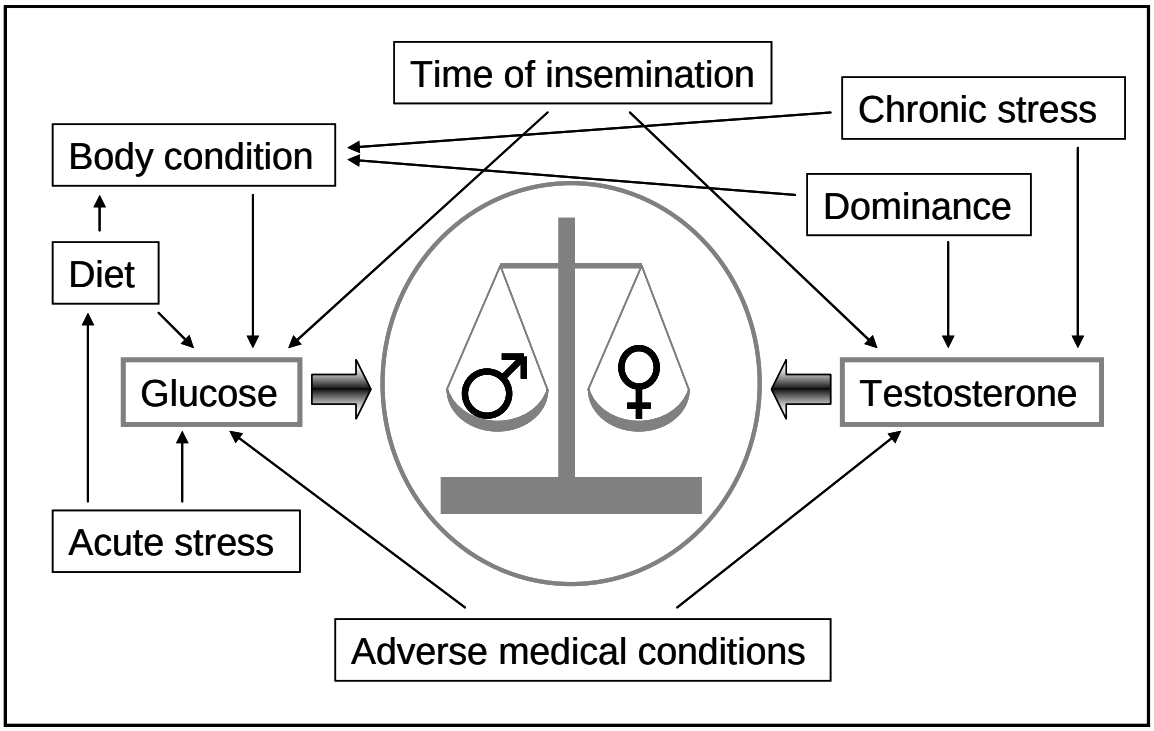


Figure 1: Relations between different maternal features suggested to affect the sex ratio and the two physiological markers proposed to explain the discrepancies between studies.

Preconceptional mechanisms

Before conception, sex ratio control mechanisms must involve either the oocyte or the spermatozoa. In the first case, they would imply a putative spermatozoa selection mechanism carried out at the site of fertilization by the cumulus cells or the zona pellucida, whereas the second could be produced by differences in motility, viability, acrosome reaction, mortality or interaction with the female genital tract, between X- or Y-bearing spermatozoa, which would lead to different chances to reach the fertilization site.

The hypothesis of a putative preferential selection of X- or Y-bearing spermatozoa was firstly suggested by Dominko et First [169] and requires the presence of sex-specific proteins in the membrane of the spermatozoa which would allow the cumulus-oocyte complex to distinguish them. The existence of these sex-specific proteins is theoretically possible due to post-meiotic transcription [170-174]. On behalf of this hypothesis, maturation stage of the oocyte matured *in vitro* has been found to exert an influence upon sex ratio in bovine [64, 169, 175]. In particular, it

was found a positive correlation between maturation time and sex ratio, which provides a mechanism for the increased sex ratios observed after late insemination [64-66]. This particular issue will be addressed in chapter 1. In the same trend, the level of testosterone in the bovine follicular fluid has been positive correlated with the likelihood of the oocyte from being fertilized *in vitro* by a Y-bearing spermatozoa [176], which fits with the increased sex ratio observed in dominant mothers [54, 56]. The possibility of an oocyte sex selection has great implications when using sex-sorted sperm in animal breeding, as if oocytes are pre-determined to be fertilized by X- or Y-bearing spermatozoa, fertility rates will be low irrespective of the advance of the sorting techniques [177]. This hypothesis will be tested in chapter 2.

Differences in motility [178], migration in cervical mucus [179], capacitation rate [65], and survival [180] between X and Y-bearing spermatozoa have been proposed to exert an influence on sex ratio. Based on these differences, several sperm separation methods have been developed, but all have failed to produce consistent results and sex-sorted by flow cytometry remains as the only reliable method, as it will be discussed below. Y-bearing spermatozoa were suggested to be more motile than X-bearing in human [178], although a posterior study, which analyzed X- and Y-sorted sperm, concluded that Y bull sperm do not swim faster than X sperm in simple salt solutions, although it displayed higher linearity and straightness of path than X [181]. In agreement with a higher motility for Y-bearing spermatozoa, Kochhar et Kochhar found that a reduced coincubation time (6 h) in bovine IVF caused a reduction of the cleavage rate and an increase in the sex ratio [182]. Similar findings were obtained by Kiwata et al., although the sex ratio skew was smaller [183]. Both studies found no sex ratio skew when coincubation was prolonged, which suggests that X-bearing spermatozoa survive longer. Consistently, 24 h preincubation of bovine sperm prior to IVF was found to reduce sex ratio [180]. In contrast, a slightly longer survival for human Y-bearing spermatozoa was reported in an *in vitro* study [184].

The absence of motility differences found in simple saline solutions [181] does not exclude the possibility that they may arise in physiological conditions. Conductivity of vaginal secretions in bovine has been reported to affect sex ratio [65]. The authors described a strong relation between conductivity, sex ratio and time of

onset of estrus: early in estrus (20 h before ovulation) conductivity was low and more heifers (92.9 %) were obtained, whereas later (10 h before ovulation), conductivity increased and more bull calves were obtained (91.7 %). The authors hypothesized that Y-bearing spermatozoa capacitate earlier than X-bearing. Thus, in early inseminations, Y-bearing spermatozoa capacitate before and lose their ability to fertilize before reaching the oocyte, whereas in late insemination early capacitation is a fertilization advantage [65]. Unfortunately, although the inverse relation between resistance (conductivity) of vaginal secretions and estrogen concentration has been described [185], the extreme sex ratio skew has not been confirmed in other studies. Similarly, a possible link between sex ratio and the cervical mucus state variations caused by the changing hormonal environment before conception has been proposed for humans [186]. Increased production of estradiol tends to increase penetrability of the cervical mucus over the six days before ovulation, whereas increased production of progesterone by the corpus luteum after ovulation quickly reduces the penetrability of cervical mucus. Earlier non-fertile inseminations also reduce penetrability by littering the passages through mucus with spermatozoa, leukocytes and other debris. Assuming the previous statement and that Y-bearing spermatozoa may be better able to pass through the cervical os, the author proposed that sex ratio at conception is positively associated with the coital rate and does not change with the time of successful insemination when coital rates are high [186]. In the same line, bicarbonate is known to have a role on sperm capacitation and sperm-oocyte interaction [187], and thus the increased sex ratio observed for late matings in hamster was explained as a result of the decrease in vaginal pH [62].

Sex selection of sperm

Although more than 100 patents exist that claim successful sexing of sperm, most procedures are no more efficacious than folk methods from more than two millennia ago [188]. Nowadays, sorting by flow cytometry remains as the only reliable method, as alternative approaches have failed to success [189, 190].

X- and Y-bearing spermatozoa are known to differ in their weight and DNA content, which avoid their separation by flow cytometry. Early attempts to separate spermatozoa were based on a theoretical difference in electrical charge due to the

differences in DNA content or to hypothetical membrane differences. However, in bovine, sperm separation by electrophoresis [191] or water insoluble newtonian gels [192] failed to alter sex ratio. The differences in DNA content leads to a difference in spermatozoa weight which inspired methods based on sedimentation or density gradients such as Percoll, albumin, Sephadex or glass-wool. Certain Percoll gradients were reported to alter sex ratio in human [193-195] and to cause a slight change in bovine [196], but other studies in human [197, 198] or bovine [199-201] failed to repeat these results. Similarly, certain albumin gradients were reported to select Y-bearing spermatozoa in bovine [202] and human [203], although other groups failed to repeat these results in rabbit [74], bovine [144, 204] and human [143, 205-209], and even lower sex ratios have been reported in human [210]. Sephadex columns [208, 211] and glass-wool in humans [198], and sedimentation methods in rabbit [212, 213] have also failed to alter sex ratio.

Methods based on putative differences in motility, pH susceptibility and membrane proteins have lead to negative or inconsistent results. In bovine, double Swim-up was reported to increase the percentage of Y-bearing spermatozoa [214], but no sex selection was found in human [195, 198, 215-217]. In rabbit, pH change of the seminal plasma failed to alter sex ratio [218]. Preliminary success was obtained by immunological sexing of sperm, based on hypothetical sex-specific proteins (SSPs) present in the spermatozoa [219] and was reported to success in bovine [220], however, other groups failed to detect those sex specific antigens [171, 221, 222].

Flow cytometric sperm sorting is based on differences in their DNA content, indirectly measured by the fluorescence emitted by the DNA-binding dye Hoechst 33342. Since the first report of sexing by flow cytometry [223, 224] and the first offspring obtained by sexed sperm [225, 226], flow cytometer/sorters have improved, especially in terms of speed of sorting [188, 227], and nowadays bovine sex-sorted semen is widely commercially available. Despite United Kingdom being the first country to effectively commercialize sex sorted semen [11], currently it is more extensively produced and used in USA for both commercial and productive reasons, such as the higher demand for replacement heifers compared to Europe [228]. In USA sexed semen inseminations in heifers accounted for 1.5, 9.6 and 14.2 % of all reported breedings for 2006, 2007, and 2008, respectively, whereas for cows, they account for

0.1, 1.3 and 2.1 %, thus showing an increase in usage [229]. Although accuracy of sexing by flow cytometry is around 90 % in most species, including cattle [228, 230, 231], this technology is rather expensive for the farmer and the semen facility. Yearly operating cost for the first year of a sex-sorting facility may exceed US$ 2 million [11], and regarding to the farmer, apart from the higher cost per seminal dose, the use of sorted semen in farm conditions usually leads to a reduction in fertility (review in [232]). The three most influencing factors affecting the profitability of the use of sex sorted semen are the differences of worth between male or female offspring, and the differences in price and fertility between the unsorted or sorted semen doses. However, it is difficult to take into account all the factors that could account for the economic outcome of the use of sorted semen, such as accuracy of sexing (which is normally around 90 %), the cost of the insemination failure, advantages of reduced dystocia in heifers pregnant with a heifer instead of a male calve [233], increasing milk production due to the lower weight of the female fetus [234], genetic gain from sexed sperm [235], or opportunities to exploit alternative enterprises and strategies [11]. After made some assumptions, it has been suggested that calves of the more valuable sex must be worth at least 200 US$ more than those of less valuable sex for sexed semen to make economic sense [11].

Among the different factors affecting the economic profit for the use of sorted semen, reduced fertility of sexed sperm will result in considerable deficits [11]. Using sorted semen, conception rates at first service average in USA 47 % for Holstein heifers and 53 % for Jersey heifers, which are around 80 % of that achieved with conventional semen [230]. In an European production system, specifically in Denmark, conception rate using sorted semen was 5 % lower than with conventional doses for Danish Reds (60.2 %), 7 % lower for Jerseys (46.6 %) and 12 % lower for Holsteins (49.3 %) [231]. Similar results were found in The Netherlands by Frijters et al., who report a 13.6 % decline in non-return rate in Holsteins, and attributed two-thirds (8.6 %) to the low dosage and a third (5 %) to the process of sorting [236]. Several factors have been proposed for the reduced fertility rates, including lower doses of spermatozoa used [237], damage to the spermatozoa caused by the sorting procedure [238] and a putative spermatozoa selection by the oocyte [176]. These hypotheses will be treated in chapters 2 and 3.

Although normal progeny is obtained by sorted sperm (reviewed in [239]), it has been suggested that damaged spermatozoa may be able to fertilize oocytes, resulting in low-quality embryos [240]. Consistently, two reports have found abnormalities in gene expression [241] or ultrastructure [242] in embryos produced with sex sorted spermatozoa, which may lead to embryo mortality. According to the farm data available, any increase in embryonic mortality due to the use of sexed semen is likely to occur early enough that the period of maternal recognition is not affected, and thereby a high percentage of females returns to estrus at normal inter-estrus intervals [230], which may be also consistent with the absence of fertilization. However, low-quality embryos may develop to term giving rise to long-term effects in the offspring [243]. The possible effect of sorted semen on embryo quality, measured in terms of gene expression and speed of development, will be analyzed in chapter 3.

Despite sex sorted technology being mainly used in cattle, the technology has been applied to a variety of mammalian species including rabbit [226], cat [244], dog [245], swine [246], horse [247], ovine [248], non-human primates [249], and human [250]. In human, the use of sorted sperm for sex selection constitutes an alternative to embryo biopsy which avoids the possible negative effects of the embryo manipulation and the destruction of embryos of the undesired sex. Thereby, there is an increasing interest in using sorted sperm as a preconceptional method of sex selection that could be employed to reduce the risk of a sex-linked disease or to balance the sex distribution of children in a family [251]. Sorted semen technology is also useful for wildlife management and conservation [252].

Postconceptional mechanisms

Sex ratio may be modified by selective loss of embryos or fetuses of one determined sex. These mechanisms, unlike preconceptional mechanisms imply a loss of fertility and reproductive success and, therefore, are more likely to occur in polytocus species which produce abundant offspring, each of which has a relatively low probability to survive to adulthood (r-selected species) rather than in those species which invest more heavily in fewer offspring (k-selected species). For r-selected species, the selective loss may occur even after birth, as it was suggested by Trivers and Willard [15], and confirmed in some rodents [83, 253, 254].

Among the sex ratio skews reported, postconceptional mechanisms may explain those which were accompanied by a reduction in fertility, especially those observed under adverse or stressful conditions. These sex ratio skews under adverse or stressful conditions may occur for both r- or k-selected species. Although under *in vitro* conditions a preferential loss of female embryos have been observed in bovine [255], male embryos and fetuses are though to be more sensible to adverse conditions than their female counterparts. In humans, there is a higher loss for male fetuses than female [256]. In agreement with the lower sex ratios found under maternal exposure to contaminants, several studies report a higher susceptibility of male fetuses compared with female to environmental contaminants, such as pesticides [257, 258]. Similarly, maternal smoking was found to delay fetal growth to a greater extend in males than in females [259], although the antioestrogenic effect of tobacco also provides a link between smoking, low levels of testosterone and low sex ratios [260]. A higher mortality for male fetuses compared with female have been suggested to be the cause for sex ratio skews caused by stress [261], and female biased sex ratios have been found to occur as a result of preferential male fetal loss in rat [262] and hamster [91].

The earlier the selective loss of embryos/fetuses occurs, the less the reproductive success and the maternal energetic reserves will be affected. When the selective loss occurs early in the preimplantation period, before maternal recognition, the female returns to estrus at normal inter-estrus intervals and her energetic loss is comparable to a non-fertile insemination. Selective loss during these early developmental stages implies the existence of a sexual dimorphism occurring before gonadal differentiation, which -as it will be discussed below- has been demonstrated. Total glucose metabolism was found to be two-fold higher in males compared with females, and the activity of the pentose phosphate pathway (PPP) is four times greater in female than in male bovine blastocysts [263]. Higher pyruvate and glucose uptake were also found for male human embryos [264]. In the same tendency, in bovine IVC, it has been observed a positive correlation between glucose concentration and sex ratio [265] and that glucose accelerates the development of males and slows down the development of female embryos [266, 267]. Transcriptional studies have found a higher expression of the X-linked gene glucose-6-phosphate dehydrogenase (*G6PD*) in bovine [268-270] and human [271], which may be responsible for these metabolic

differences. G6PD is the first and key PPP regulatory enzyme, being essential for providing NADPH-reducing power, which provides a link between stress response and transcriptional sexual dimorphism that may explain why female embryos survive better under oxidative stress conditions in mice [272]. Consistently, it has been proved that G6PD inhibition removes some of the sexual dimorphism in mice [272] and bovine [265]. G6PD has been also proposed as a candidate to explain the sex ratio skews caused by diet (reviewed in [266]). In an *in vivo* approach to determine glucose role on sex ratio control, dexametasone treatment in mice was found to decrease both sex ratio and glucose levels [273]. Similarly, glucosamine addition to the culture medium after the 8-cell stage was found to decrease embryo development rates and skew the sex ratio in favor of males [274]. Furthermore, the effect was negated by the addition of an inhibitor of O-linked GlcNAc transferase (OGT), an X-linked enzyme involved in glucosamine metabolism [274].

Preimplantational sexual dimorphism

Preimplantational sexual dimorphism provides a physiological basis for the different survival rates of male and female embryos observed under adverse conditions. However, this is not the sole implication of the preimplantation sexual dimorphism. The study of this phenomenon in early stages of development will help to understand phenomena such as sex chromosome transcriptional regulation, early X-chromosome inactivation – which has been thoroughly studied only in mouse -, early sex determination, sex chromosome evolution, X-linked diseases, and sex-specific long-term effects regarding to the embryonic origin of adult diseases.

Early studies supported the idea that embryos develop in a non-sexspecific manner until the develop of the gonads [275]. However, now it is known that preimplantation embryos displayed sexual dimorphisms that occurs before gonadal differentiation and can only be attributable to their differences in sex chromosome dosage [276]. Apart from the differences in glucose metabolism previously described, in eutherian mammals, male embryos have been suggested to survive better after vitrification [277], and to develop faster than their female counterparts before sexual differentiation. Thus, sex related differences in speed of development have been reported in mouse [278], swine [279], ovine [280], bovine [255, 281-284], and human

[285]. If this notion was true, a skew of the sex ratio would be expected following *in vitro* techniques, as in both humans and farm animals, the faster embryos are considered to be of better quality and thus, are selected to be transferred. However, in human, no sex ratio skew was observed after the transfer of fast developing embryos [286, 287]. Consistently, no sex related differences in speed of development have been observed in mice [16, 288], rats [84], swine [289, 290], ovine [43] and bovine [288, 291]. Suboptimal *in vitro* conditions may be the cause for the differences in speed of development which may lead to a sex-specific embryo loss or selection. Thus, in mouse, it was observed that the acceleration of male preimplantational development occurs *in vitro* but not *in vivo* [292]. Also in bovine, increased sex ratio at the expanded blastocyst stage was found for *in vitro* cultured embryos, but not for those cultured in the sheep oviduct [293], and, as previously noted, the presence of high concentration of glucose cause sex-related differences in speed of development, which are not observed at optimal levels [267]. Absence of differences was also found in bovine embryos cultured without serum [294]. In human, embryo transfer at early stages did not skew the sex ratio [295], whereas there are several reports for a sex ratio skew in the offspring when embryos are transferred at the blastocyst stage [296-298]. In the same tendency, it has been reported that only male embryos derived by ICSI, but not those derived by IVF, grow faster than their female counterparts [299]. Consistently with a putative higher speed of development, *in vitro* produced male embryos have been found to content more cells than female in bovine [255] and human [285]. Also in human, third trimester male fetuses were bigger than female, which was attributed to differences in chromosome constitution [300]. However, other studies found no differences among sexes in bovine [284, 301]. Preimplantation sexual dimorphisms in terms of different survival in *in vitro* culture conditions and speed of development will be addressed in chapters 1 and 3.

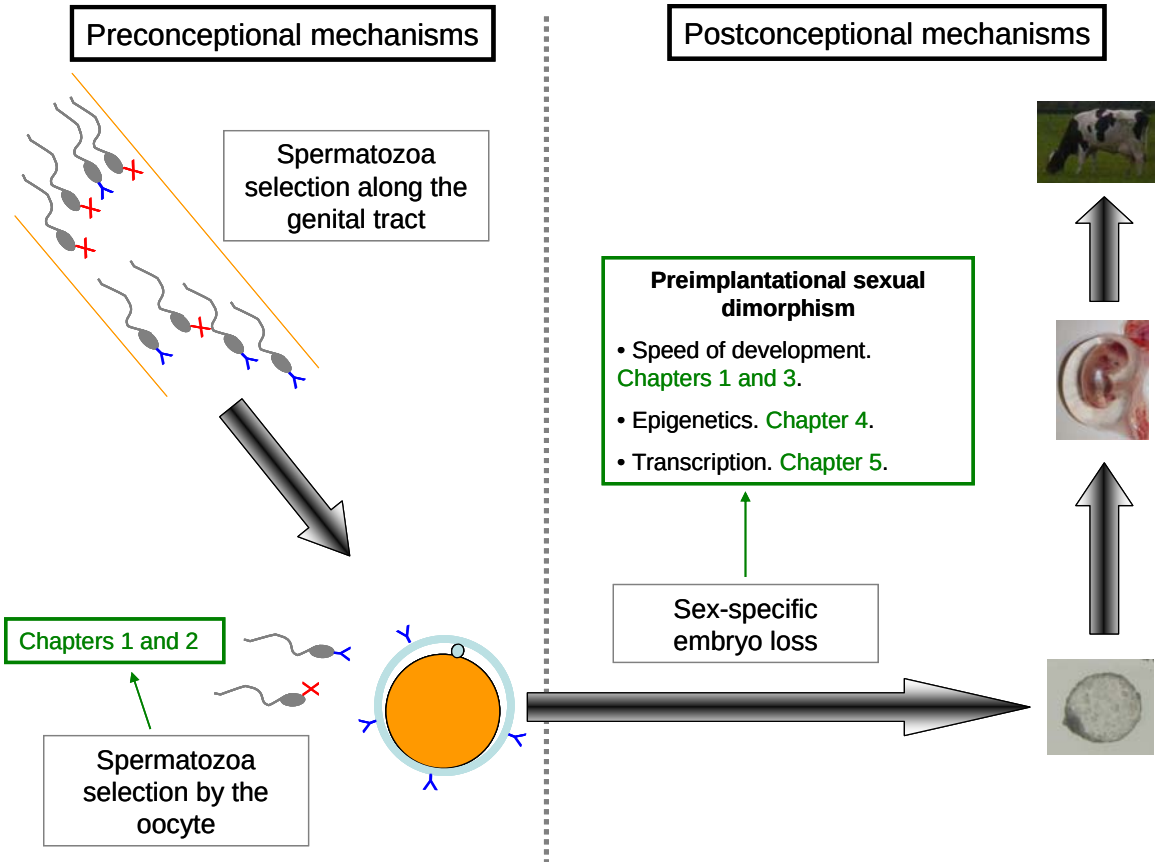
Preimplantation sexual dimorphism is the consequence of a transcriptional dimorphism originated by the differences in sex chromosome dosage. Y-linked genes are male exclusively expressed and thus, the first suggestion for sex related transcriptional differences in preimplantation embryos was described for histocompatibility Y antigen (Hya), which was present in 50 % of the eight-cell-stage mouse embryos [302], which were later confirmed to be males [303]. Later, SRY transcription was reported for male embryos in mouse [304, 305], bovine [306] and

human [307]. X-linked genes are present in double dose in females, but dosage compensation by random X-chromosome inactivation ensures an equal transcription level of most X-linked genes for both sexes in adult tissues. However, during the preimplantation period, X inactivation is a reversible dynamic process [308] and some genes escape from inactivation and are expressed at a higher level in female embryos, as it has been reported for mouse [309-312], bovine [241, 268, 269, 313] and human [271] embryos. The extent of this escape from inactivation remains unclear. Apart from this direct effect on gene expression, genes allocated on sex chromosomes modulate the expression of autosomal-linked genes, leading to sex related differences [241, 314]. Among the few autosomal-linked genes reported to displayed sex-related differences in their expression patterns, IFN-Tau (IFNT) -which signals pregnancy to the mother in ruminants [315]- has been widely studied. Female bovine blastocysts produce more IFNT than male in both *in vitro* [314] and *in vivo* conditions [316], although the differences disappeared in day 14 elongated embryos [316]. Similarly, female day 13 ovine conceptuses produce slightly more IFNT than males [43] and in red deer (*Cervus elaphus*) IFNT was found in 60 % of 10 female blastocysts whereas it was not found in 7 male blastocysts [19]. These differences may be linked to glucose metabolism, as inhibition of G6PD in bovine embryos suppresses the differential expression of *IFNT* [317]. The sexual dimorphism in the transcription of selected candidate genes will be addressed on chapters 3 and 4. Most of the studies which have analyzed preimplantation transcriptional dimorphism are based on a small number of candidate genes. Global gene expression studies provide an estimation of the extent of the transcriptional sexual dimorphism, which helps to understand phenomena such as sex chromosome transcriptional regulation, X-chromosome inactivation and sex ratio skews due to postconceptional mechanisms. However, due to the technical difficulties in obtaining the necessary large number of embryos per group to perform the molecular biology techniques, there is only one report which analyzes global gene expression sex-related differences, which was performed in the mouse model [318]. In chapter 5, global gene expression sex-related differences will be analyzed in the bovine model.

Epigenetics is a term in biology used today to refer to features such as chromatin and DNA modifications that are stable over rounds of cell division but do not involve changes in the underlying DNA sequence of the organism [319].

Epigenetics controls DNA transcription and constitutes the way the genome integrates intrinsic and environmental signals [320]. Epigenetic status, especially DNA methylation or histone methylation or acethylation levels, is the basis for transcriptional differences and thus, transcriptional sexual dimorphism should be originated by epigenetic events. However, during preimplantation development, X-chromosome inactivation remains as the only sex-specific epigenetic difference reported. Apart from DNA and histone modification, there are other epigenetic features, such as mitochondrial DNA copy number and telomere length. Mitochondria are responsible for oxidative glucolysis, whose importance as a significant source for ATP production increased during the preimplantation period [321-323], coinciding with an increase in mitochondria maturation [324] and number [325]. Due to their importance in two factors affected by preimplantational sexual dimorphism (i.e. embryo metabolism and oxidative stress [272]), mitochondria has been proposed to play an important role in sex-related differences [326]. Telomeres are repeated DNA sequences situated at the end of the chromosomes, whose length determines the limit of divisions an adult somatic cell can support before senescence [327]. Telomere length is determined in the preimplantation period, when telomeres elongate by an alternative recombination-based mechanism [328] followed by telomerase [329]. On chapter 5, sex-related epigenetic differences at the blastocyst stage in terms of gene expression of epigenetic related enzymes, methylation status of repeated sequences, mtDNA copy number and telomere length will be determine.

Figure 2: Putative sex ratio control mechanisms and their relations with the chapters of this thesis.



INTRODUCCIÓN

El sexo es la característica individual que más influye en el fenotipo de los mamíferos. El género de la descendencia humana y animal ha sido una de las preocupaciones más recurrentes de la humanidad a lo largo de la historia. Como ejemplo de este interés, el sexo es lo primero que conocen nuestros padres de nosotros, el género es la única característica genética indicada en los documentos de identidad de distintos países y en la mayoría de las lenguas, los sustantivos tienen un género. Por ello, no sorprende que la determinación del sexo de la descendencia de los mamíferos haya sido objeto de estudio desde la antigüedad [1].

Las distintas culturas han confiado en diversos métodos de control del sexo de la descendencia. Los filósofos griegos pensaron en un principio que el origen de la determinación del sexo se hallaba en la lateralidad, basándose en la asociación del lado derecho con la bondad, el sol, el calor y el hombre, y del lado izquierdo con la maldad, la luna, el frío y la mujer. Parmemides (515 A.C. aprox.) propuso que el sexo del embrión estaba determinado por el lado del vientre en el que se desarrollara, mientras que Anaxágoras (500 a 419 A.C.) expuso que el testículo derecho daba lugar a la concepción de machos y el izquierdo a hembras, proponiendo la ligadura del testículo izquierdo para concebir hijos [1]. La idea se mantuvo en el "*Corpus hippocraticum*" (siglos V y IV A.C.), y los textos tántricos hindúes (siglos VII a XVII) introdujeron una variación, declarando que si en el momento del orgasmo, el "aliento solar" tomado por la ventana derecha de la nariz dominaba en el hombre y el "aliento lunar" tomado por la ventana izquierda de la nariz dominaba en la mujer y tenía lugar la concepción, se obtendría un niño, mientas que lo opuesto daría lugar a una niña [2]. En la nobleza francesa se seguía aconsejando la extirpación del testículo izquierdo para concebir varones [3], y Millot, el tocólogo de la reina María Antonieta de Francia, escribió "*es el último movimiento de la mujer el que determina el sexo del bebé: es el lado sobre el que reposa en el momento de la eyaculación el que determina el sexo del bebé: siempre un niño cuando es hacia el lado derecho y siempre una niña cuando es hacia el lado izquierdo*" (citado en [4]). Aristóteles (384-322 A.C.) criticó las teorías de Parmemides y Anaxágoras en base a la evidencia de que se pueden encontrar embriones de ambos sexos en el mismo lado del útero y que los hombres con un único testículo tienen descendencia de ambos sexos [1] y propuso

que la probabilidad de tener un varón se correlacionaba directamente con el vigor mostrado durante el coito [2]. Empedocles (494-434 A.C.) indicó que el momento de la cópula podía afectar a la proporción de sexos debido a las diferencias en "cantidad de calor", y la idea se ha mantenido en la actualidad en el método de Shettles [5].

En la cultura hebrea, el Talmud (siglo V A.C.), basado en *Leviticus 12:2*, afirma que un orgasmo femenino anterior al masculino daba lugar a descendencia masculina [4]. El Talmud también aconsejaba situar el lecho matrimonial en una dirección norte-sur para favorecer la concepción de varones [4]. Las culturas asiáticas creían en una influencia de la astronomía sobre la proporción de sexos. En Corea del Sur, la tradición considera que el año del caballo trae consigo circunstancias desfavorables que dan lugar al nacimiento de más niñas [6]. La tabla china de selección del género emplea la edad lunar de la mujer y el mes lunar de la concepción para predecir el sexo de la descendencia. Los textos indios ayurvédicos de Sushruta (siglo V A.C.) y Charaka (siglo II A.C.) sugieren el uso de distintas hierbas en combinación con eventos astrológicos a los dos meses de gestación con el fin de aumentar las probabilidades de engendrar un varón [7]. La noción se ha mantenido a lo largo del tiempo y actualmente en la India rural se siguen utilizando preparados basados en plantas como la bryonia laciniosa (*Bryonia laciniosa*) y el roble del Kurdistán (*Quercus infectoria*) [7]. El español Huarte San Juán reunió distintos métodos tradicionales en su libro "*Examen de ingenios para las ciencias*" (1575), que incluía varios consejos para el hombre relacionados con la dieta, el ejercicio y el momento y la posición del coito. Ciertos métodos simbólicos, como la tradición germana que aconsejaba al hombre llevar un hacha a la cama para concebir varones [4], se han usado en Checoslovaquia y Hungría, Islas Palau, Yugoslavia e Italia [2].

A finales del siglo XIX, L Cuénot en su libro "*Fin de siècle*" comentaba acerca de los métodos de control de la proporción de sexos de la descendencia: "*Es ciertamente humillante reconocer que en lo relativo al hombre y otros mamíferos no se ha avanzado desde los tiempos de los predecesores de Aristóteles*" (citado en [1]). El descubrimiento del papel de los cromosomas sexuales en la determinación del sexo de los mamíferos constituyó un gran avance [8] que desafió todas las teorías postconcepcionales. En el siglo XX, el método Shettles, descrito en el libro "*How to choose the sex of your baby*" [5], es probablemente la estrategia natural más conocida.

Está basado en la supuesta diferencia en motilidad y supervivencia entre espermatozoides X e Y, y afirma que adoptando ciertas posturas sexuales, el momento del coito en función del ciclo menstrual determina el sexo del bebé. La comunidad científica ha criticado seriamente la eficacia del método [9], y en la actualidad, sólo tres métodos han demostrado su capacidad para alterar la proporción de sexos de la descendencia: el uso de semen sexado, la determinación del sexo mediante biopsia embrionaria y las modificaciones transgénicas [10]. A pesar de esto, una búsqueda en internet de métodos alternativos encontrará distintos consejos y productos para obtener descendencia de un sexo predeterminado, incluyendo diferentes dietas, ingestión de café o chocolate, "nutraceúticos especialmente equilibrados", temperatura testicular, pH vaginal, orgasmo, postura sexual, posición de la luna con respecto a las constelaciones, fase lunar, fechas específicas… y el libro de Shettles se publicó en su 6ª edición en 2006.

El sexo está considerado como el carácter genético más importante en la producción animal [11], ya que ejerce una gran influencia sobre las tasas de crecimiento, la producción lechera y la susceptibilidad a enfermedades. Como carácter genético, el sexo constituye una excepción que sólo puede ser manipulada mediante métodos de predeterminación del sexo y no por selección genética. Algunos estudios han determinado los beneficios económicos de un sistema de predeterminación del sexo en la producción animal. En el vacuno lechero, sólo las terneras son productivas, puesto que los machos presentan un pobre rendimiento productivo cárnico. De este modo, se ha estimado que si se pudiese seleccionar la descendencia hembra en el momento de la inseminación, la eficiencia de la producción lechera podría aumentar un 30 % [12]. De forma similar, en el vacuno cárnico, se ha calculado que el beneficio neto se incrementaría en un 20 % si el porcentaje de machos aumentase de un 50 a un 90 % [13].

La proporción de sexos se define como la proporción de descendencia macho con respecto a la descendencia hembra. La espermatogénesis transforma la espermatogonia diploide en espermatozoides haploides, dando lugar a la misma cantidad de espermatozoides X e Y. Asumiendo que la supervivencia y transporte en el tracto genital femenino y la capacidad de fecundación es igual para los espermatozoides X e Y, y que ambos sexos sufren la misma mortalidad, la proporción

de sexos debería ser 1:1. La proporción de sexos se puede medir en distintos puntos de observación: la proporción de sexos primaria es la relación de sexos macho-hembra en el momento de la concepción, la secundaria se mide en el nacimiento, la terciaria se determina en la pubertad y la cuaternaria se observa al final de la edad reproductiva. Bajo circunstancias normales, todas las especies mamíferas muestran una proporción de sexos secundaria aproximadamente similar a la equidad (1:1). Sin embargo, ciertos estudios han observado una interacción entre la proporción de sexos y ciertas características o condiciones maternas o paternas. El término proporción de sexos “aumentada” o “alta” se refiere a una variación significativa de la proporción de sexos teórica a favor de los machos, mientras que lo contrario (“disminuida” o “baja”) indica una proporción de sexos sesgada a favor de las hembras. Una variable que muestra una “correlación positiva con la proporción de sexos” la aumenta, mientras que una “correlacionada negativamente” la disminuye.

Sesgos de la proporción de sexos originados en la hembra

Dado que la contribución de la hembra mamífera al cuidado de su descendencia es mayor que la del macho, antes y después del parto, tiene sentido pensar desde un punto de vista evolutivo en una aportación materna a la proporción de sexos [14]. Se ha indicado que algunas características maternas, como la condición corporal, la dieta, los niveles de glucosa, el estatus de dominancia, los niveles de testosterona, el estrés, la edad, el número de parto y el tamaño de camada, y algunos parámetros ligados a la hembra como el momento de inseminación, el lado de ovulación, la exposición a contaminantes y a temperatura ambiental, pueden modificar la proporción de sexos.

Entre estos factores, el estado nutricional de la madre ha suscitado un interés especial. Una teoría especialmente aplicable a especies polígamas, conocida como la hipótesis de Trivers y Willard, sugiere que conforme disminuye la condición de la madre, la hembra adulta tiende a producir una proporción macho-hembra más baja, porque se espera que un macho en buena condición se reproduzca más durante el periodo de inversión parental que una hermana en condiciones similares, mientras que es previsible que una hembra en una condición pobre se reproduzca más que su hermano en la misma condición [15]. Además, parece lógico que las madres en una

condición pobre produzcan una descendencia con predominio de hembras, ya que la hembra requiere más recursos para desarrollar un feto macho que una hembra. Ciertas evidencias relacionan un plano alto de nutrición materna con un aumento de la proporción de sexos (revisado en [16]). Algunos estudios retrospectivos llevados a cabo en ballenas jorobadas (*Megaptera noraeangliae*) [17], ciervos europeos (*Cervus elaphus)* [18, 19], renos (*Rangifer tarandus*) [20], equinos [21], bovinos [22] y humanos [23], han asociado un predominio de la descendencia macho con una buena condición materna, mientras que se ha observado un sesgo a favor de las hembras en madres con bajo peso o bajo circunstancias desfavorables en equinos [21], ratas [24], y en humanos en el primer [25, 26] y segundo [26] parto. Sin embargo, no se encontró un efecto de la condición maternal sobre la proporción de sexos en carneros de las Rocosas (*Ovis canadensis*) [27]. Más que la condición corporal *per se*, se ha propuesto que el cambio en la condición corporal puede ser el mecanismo causante del sesgo de la proporción de sexos mediante la concentración de glucosa. En este sentido, se ha descrito que las yeguas que aumentan su condición corporal exhiben un sesgo extremo a favor de los machos en su descendencia, mientras que lo opuesto se observó en aquellas que perdían condición [28].

Suponiendo que la condición nutricional altera la proporción de sexos de la descendencia, sería posible un control nutricional de la misma. En la misma línea de un predominio de machos en la descendencia de madres en buena condición, se ha observado que las dietas altas en calorías aumentan la proporción de sexos en zarigüeyas (*Didelphis marsupialis*) [29] y gamos (*Dama dama*) [30]. De forma similar, en vacas lecheras, aunque no en terneras, se ha visto que un alto plano nutricional da lugar a un exceso de machos en la descendencia en comparación con una dieta más pobre [31], y en humanos, la disponibilidad de calorías se ha correlacionado positivamente con la proporción de sexos [32]. Siguiendo la misma tendencia, se ha encontrado que los niveles altos de glucosa materna durante la fecundación están correlacionados con un aumento en la proporción de sexos en el topillos agrestes (*Microtus agrestis*) [33], y los ratones diabéticos dan lugar a una descendencia abundante en machos [34], aunque en humanos se ha descrito la situación opuesta [35]. En el otro extremo, una mala condición maternal se asocia con una dieta pobre que debería conducir a un predominio de hembras en la descendencia. En ratones se ha indicado que una restricción en la dieta disminuye la proporción de

sexos [36]. En la misma especie, se obtuvo una proporción de sexos 1:3 mediante una dieta baja en grasa [37], y una privación intermitente del alimento durante la semana anterior a la monta produjo un exceso de hembras en las camadas [38]. La restricción de alimento también favoreció la obtención de descendencia hembra en ratas [39]. En contraposición, en ciervos de cola blanca (*Odocoileus virginianus*), se asoció la dieta pobre con un exceso de machos en la descendencia [40]. Aunque la mayoría de los estudios se han centrado en el contenido calórico de la dieta, es posible que su efecto se deba al nivel de algunos componentes específicos. Así, una dieta isocalórica con respecto al control pero con un alto contenido graso aumentó la proporción de sexos en ratones [41]. Una dieta con una elevada proporción grasa puede dar lugar a altos niveles de glucosa circulante, lo que apoya la hipótesis de la glucosa como un factor determinante de la proporción de sexos [42]. En la misma línea, una dieta isocalórica con respecto al control, pero con un contenido aumentado en ácidos grasos poliinsaturados protegidos en el rumen aumentó la proporción de sexos en embriones ovinos de día 13 [43] sin incrementar los niveles de glucosa, llevando a los autores a proponer dos posibles efectos nutricionales distintos del efecto directo sobre el embrión: la dieta puede retrasar la ovulación y aumentar así la proporción de sexos –como será discutido posteriormente-, o puede haber una atracción de los espermatozoides Y debido a la acumulación de ácidos grasos poliinsaturados n-6 (omega 6) en el ovocito [43]. Por el contrario, una dieta con un alto contenido en ácidos omega 6 sesgó la proporción de sexos a favor de las hembras en ratones, mientras que una dieta alta en omega 3 no causó efecto [44]. Se han descrito otros componentes de la dieta que pueden alterar la proporción de sexos. Los componentes minerales fueron propuestos como alterantes de la proporción de sexos en estudios iniciales llevados a cabo en el modelo bovino (citados en [4]). En ratas se describió una relación inversa entre el contenido en sodio de la dieta y la proporción de sexos [45], y posteriormente se observó que un aumento de calcio y magnesio en la dieta combinado con una reducción de sodio y potasio disminuía la proporción de sexos, probablemente a causa de un descenso en la actividad de la enzima glicerilfosforilcolina diesterasa [39].

Otro factor que se ha asociado con alteraciones de la proporción de sexos es la dominancia materna, que también se ha relacionado con la condición materna y la dieta. Las madres dominantes se deberían encontrar en una mejor condición que sus

subordinadas y, por ello, ambos factores se asocian frecuentemente dando lugar proporciones de sexos aumentadas en madres dominantes [18, 19], aunque una "buena condición" no es siempre sinónimo de dominancia materna [46]. Se han propuesto dos hipótesis opuestas. En primer lugar, una hipótesis [47] aplicable a especies primates matrilineales declara que una madre dominante dejaría más descendientes a través de una hija que a través de un hijo si la hija heredase su alto rango, mientras que una madre de bajo rango de la misma especie obtendría más descendientes mediante un hijo, que probablemente emigraría en la pubertad y no heradaría necesariamente el bajo rango de su madre. Esta situación se ha observado en monos rhesus (*Macaca mulatta*) en cautividad [47] y en macacos coronados (*Macaca radiata*) [48]. En oposición, la segunda teoría, que tiene mayor apoyo y coincide con la condición materna, establece que una hembra dominante tiende a producir más machos en la descendencia. Varios trabajos apoyan la segunda teoría; así se ha descrito que las hembras mamíferas dominantes tienden a generar en su descendencia un mayor número de machos en poblaciones cautivas y salvajes de ciervos europeos (*Cervus elaphus*) [18], cerdos [49], arruis (*Ammotragus lervia sahariensis*) [50], monos de Gibraltar (*Macaca sylvanus*) [51] y macacos cangrejeros (*Macaca fascicularis*) [52], mientras que otras observaciones muestran que las hembras de bajo rango dan lugar a un predominio de hembras en su prole en ratones [53] y monos de Gibraltar (*Macaca sylvanus*) [51]. Una situación similar se ha observado en humanos [54], y una relación más compleja entre edad materna y dominancia se observó en papiones (*Papio cynocephalus*): las hembras subordinadas parieron más hijos siendo jóvenes y las dominantes más hijos siendo viejas [55]. La dominancia está relacionada con los niveles de testosterona maternos (revisado en [56]). En este sentido, se han asociado altos niveles de testosterona materna con proporciones de sexo aumentadas en bovinos [56] y topillos agrestes (*Microtus agrestis*) [33], y dos parámetros afectados por las concentraciones de testosterona y estradiol -la proporción cadera/cintura y la facilidad de orgasmo- se han correlacionado positivamente con la proporción de sexos en la especie humana [57]. En desacuerdo con ambas teorías, dos metaanálisis han observado que el efecto de la dominancia maternal sobre la proporción de sexos en la descendencia es particularmente inconsistente a lo largo de la literatura [42, 58], y algunos estudios llevados a cabo en papiones (*Papio cynocephalus*) [59] y monos rhesus (*Macaca mulatta*) [60] no han logrado establecer una relación. Esto ha llevado

a pensar que los efectos observados pueden deberse a la variación estocástica que sucede en muestras pequeñas [58].

Uno de los factores ampliamente asociados a las alteraciones en la proporción de sexos es el momento de la monta o la inseminación en relación con la ovulación (revisado en [61]). La idea fue mencionada por primera vez por Empedocles en el siglo V A.C. y se ha mantenido hasta la actualidad en el método Shettles [5]. Existen estudios que asocian la inseminación temprana con proporciones de sexos bajas y las cópulas tardías con un aumento en la proporción de sexo en hámsteres [62], ciervos de cola blanca (*Odocoileus virginianus*) [63], ovinos [64] y bovinos [65, 66]. En contraposición, se ha observado un descenso en la proporción de sexos en cópulas tardías en ratones [67], bovinos [68] y humanos [69, 70]. Otros estudios describen una cinética en forma de U invertida, con bajas proporciones de sexos en inseminaciones tempranas y tardías en bovinos [68] y en humanos realizando inseminación artificial [71]. La forma de U se ha descrito en ratas [72] y humanos [73]: se vió una frecuencia desproporcionadamente alta de hembras en la mitad de la ventana de fertilidad y de machos en cualquiera de sus extremos. Esta tendencia también se observo en inseminación natural [71]. Sin embargo, otros estudios no han encontrado una relación entre el tiempo de inseminación en relación con la ovulación y la proporción de sexos en conejos [74], suidos [75], bovinos [61, 76, 77] y humanos [9].

Otro de los factores que desde la antigüedad se ha pensado que puede influir en la proporción de sexos es el lado en el cual tiene lugar la fecundación-gestación. Parmemides fue el primero en ofrecer la idea en el siglo VI A.C., asociando el lado derecho con la descendencia masculina y el izquierdo con la femenina, como un mecanismo de determinación del sexo. En la actualidad la idea se mantiene, aunque la explicación moderna es una supuesta selección del ovocito de los espermatozoides X o Y. De acuerdo con Parmemides, se ha publicado que la proporción de sexos en terneros gestados en el cuerno derecho es significativamente más alta que en el cuerno izquierdo [78, 79]. De forma similar, en gerbillos de Mongolia (*Meriones unguiculatus*) se obtuvieron significativamente más machos del ovario derecho transplantado (en el cuerno derecho o izquierdo) que del ovario izquierdo transplantado [80]. Sin embargo, la situación opuesta (un aumento de la proporción de sexos en el cuerno izquierdo) se ha observado en conejos [81] y ratones [82], otros

estudios no encontraron diferencias en ratones [83], ratas [84], y cerdos [85] –aunque en esta última especie es frecuente la migración uterina-, y la idea de que el ovario de origen puede influir sobre el sexo de la descendencia se ha ridiculizado [86]. En una línea similar a la localización del embrión y la proporción de sexos, no se encontraron diferencias en la proporción de sexos entre tres segmentos uterinos (ovárico, medio y cervical) en ratones [87].

El estrés se ha asociado frecuentemente con una baja proporción de sexos. Las madres estresadas producen un exceso de hembras en sus camadas en ratas [88] y hámsteres [89]. Estas observaciones han encontrado apoyo en estudios mecánicos que describen un descenso en la proporción de sexos en ratas tratadas con ACTH [90] o una recuperación de la proporción de sexos normal en hámsteres estresados tratados con dexametasona [91]. En humanos también hay evidencias de una correlación negativa entre la proporción de sexos y el estrés. Por ejemplo, la proporción de sexos se ha visto disminuida después de situaciones de estrés agudas causadas por circunstancias vitales severas [92], desastres naturales, como la niebla tóxica de Londres en 1952 y las inundaciones de Brisbane en 1965 [93] y el terremoto de Kobe en 1995 [94], o por una guerra breve [95] o un ataque terrorista [96], y la cantidad de dosis diarias de antidepresivos y ansiolíticos en Suecia se ha relacionado de forma inversa con la proporción de sexos [97]. Sin embargo, en el topillos agrestes (*Microtus agrestis*), no se encontró una asociación entre la proporción de sexos y los niveles de corticosterona [33], y el estrés crónico relacionado con la competición por recursos locales se ha asociado con proporciones de sexo elevadas en marsupiales [98] y primates [99]. La misma situación se ha observado después de la guerra en humanos [100, 101]. Una posible explicación a estos resultados es la teoría de la asignación del sexo, que predice que en una población con la proporción de sexos sesgada por asignación, los padres aumentan su idoneidad ajustando la proporción de sexos de su progenie hacia el sexo menos frecuente [102]. De acuerdo con esta teoría, se detectó que en la mayoría de las 21 parroquias de la Finlandia preindustrial analizadas, en las que el 90 % de los matrimonios se concertaban dentro de cada parroquia, se tenían más hijos cuando los hombres eran más escasos que las mujeres [103]. En camadas de lobos, se observó un sesgo a favor de los machos cuando la densidad de población era alta, y una ausencia de sesgo o un predominio de hembras cuando la densidad de población era baja [104], y se han citado situaciones similares

en lemúres ratón grises (*Microcebus murinus)* [105] y papiones (*Papio cynocephalus*) [59], y en rinocerontes negros (*Diceros bicornis*) en cautiverio [106].

La edad materna, el número de parto y el tamaño de camada también pueden ejercer una influencia sobre la proporción de sexos. La edad materna se ha correlacionado negativamente con la proporción de sexos fetal en ciervos de cola blanca (*Odocoileus virginianus*): las ciervas mayores de 4,5 años concibieron más hembras y las menores de 3,5 años más machos [107]. En hámsteres, se detectó una relación curvilínea entre la proporción de sexos y la edad materna cuando se producía una única monta: las hembras cubiertas a los 100 días de edad obtuvieron más machos que aquellas cubiertas a los 55 días, pero menos que aquellas cubiertas por primera vez a los 325 días, lo que fue explicado como una consecuencia de la mayor susceptibilidad de los embriones macho a agentes estresantes en comparación con sus compañeras [108]. La relación curvilínea inversa se ha descrito en ovinos, con un exceso de corderas en ovejas jóvenes y viejas y de corderos en las de mediana edad [50]. De forma parecida, se encontró una correlación positiva entre la edad materna y la proporción de sexos en topillos agrestes (*Microtus agrestis*) [33] y en bovinos [109]. En humanos se obtuvo una correlación negativa entre la proporción de sexos y la edad en Hungría [110], Estados Unidos [111], y España [112]. Sin embargo, otro estudio no encontró diferencias en monos rhesus (*Macaca mulatta*) [60]. En lo relativo al número de parto, un análisis multivariable aplicado a 1,4 millones de nacimientos en Estados Unidos encontró una débil correlación entre el orden de nacimiento y la proporción de sexos [113] y se han obtenido resultados similares en la población de Inglaterra y Gales [114]. En hámsteres se detectó un aumento de la proporción de sexos hasta el tercer parto, a partir del cual comenzó a disminuir [108]. Sin embargo no se vieron efectos en bovinos [76, 115] y monos rhesus (*Macaca mulatta*) [60]. Por último, de acuerdo con el efecto de la condición corporal, se ha observado una correlación negativa entre la proporción de sexos y el tamaño de camada en cobayas [116] y jabalíes [117], que parece deberse a una mayor mortalidad uterina de los machos. Sin embargo, no se observó efecto en cerdos [75] y el efecto contrario se observó en el topillos agrestes (*Microtus agrestis*) [33].

Finalmente, la proporción de sexos secundaria ha sido propuesta como un centinela de los cambios ambientales [118], ya que la exposición a contaminantes

puede alterarla. Un descenso en la proporción de sexos se ha relacionado con una exposición alta a contaminantes en humanos. Se ha descubierto que la proporción de sexos en las principales ciudades italianas es baja en comparación con el resto del país, siendo más alta entre los fetos abortados. Los autores propusieron que la causa del sesgo podría ser una mayor mortalidad de los fetos machos en las grandes ciudades debida a la exposición a condiciones ambientales dañinas [119]. Igualmente, la proporción de sexos fue baja en áreas con un alto riesgo de contaminación procedente de incineradoras en Escocia [120] y, tras la exposición a altos niveles de dioxinas, la población de Seveso (Italia) experimentó un descenso en la fertilidad y en la proporción de sexos [121]. De forma similar, la ingestión materna (pero no paterna) de bifenilos policlorados redujo la proporción de sexos en la población de la región de los Grandes Lagos [122]. También se ha sugerido que el hábito de fumar tabaco aumenta [123], reduce [124] o no tiene efecto sobre la proporción de sexos [125, 126]. El fondo genético, en particular el alelo *ACP1*, puede ser el responsable de las discrepancias entre los estudios basados en el consumo de tabaco [127]. Además se ha descrito que la cafeína reduce ligeramente la proporción de sexos (61% de hembras) en el hámsteres chinos (*Cricetulus griseus*) [128], y se detectó un sesgo a favor de las hembras en la proporción de sexos en humanos expuestos a alto voltaje eléctrico en Bagdag [129]. Las proporciones de sexo bajas también se han asociado a condiciones médicas adversas (revisado en [130]), como el linfoma no-Hodgkin [131], la hepatitis [132], el uso de fármacos en tratamientos de fertilidad como el clomifeno [132], la hyperplasia congénita adrenal [133] y la infección por *Cytomegalovirus* [134, 135]; aunque se ha observado un aumento de la proporción de sexos en la hepatitis B [136], cáncer de mama [137] y tras la infección por *Toxoplasma gondii* en humanos [138] y ratones [139]. El clima también se ha asociado con la proporción de sexos. La temperatura del aire y la humedad se han relacionado positivamente con la proporción de sexos en el ganado vacuno [115] y es más frecuente el nacimiento de terneros en los meses cálidos del año [109]. En humanos se han encontrado resultados similares, naciendo más niños en los años cálidos en Finlandia [140], y se ha propuesto una influencia del mes de nacimiento de la madre y del hijo en la población franco-canadiense [141] y en Módena [142].

Tabla 1: Correlación entre distintas características maternas y la proporción de sexos.

Característica materna	Correlacción positiva con la proporción de sexos	Sin efecto	Correlacción negativa con la proporción de sexos
Condición corporal	Machos en buena condición: Ballenas jorobadas [17], ciervos europeos [18, 19], renos [20], equinos [21], bovinos [22] y humanos [23]. Hembras en mala condición: Equinos [21], ratas [24] y humanos [25, 26]. Machos cuando aumenta la condición corporal: Equinos [28].	Carneros de las Rocosas [27].	
Contenido calórico de la dieta	Machos en dieta alta en calorías: Zarigüeyas [29], gamos [30], vacas lecheras [31] y humanos [32]. Hembras en dieta restringida: Ratones [36-38] y ratas [39].	Terneras lecheras [31].	Machos en dieta restringida: ciervos de cola blanca [40].
Nivel de glucosa	Topillos agrestes [33] y ratones [34].		Humanos [35].
Dieta isocalórica alta en grasa	Grasa total: Ratones [41]. Ácidos grasos poliinsaturados: Ovinos [43].		Ácidos grasos poliinsaturados n-6: Ratones [44].
Na^+ en dieta			Ratas [39, 45]
Dominancia	Machos en madres dominantes: Ciervos europeos [18], cerdos [49], arruís [50], monos de Gibraltar [51], macacos cangrejeros [52], papiones viejos [55] y humanos [54]. Hembras en madres subordinadas: Ratones [53] y monos de Gibraltar [51].	Papiones [59] y monos rhesus [60].	Monos rhesus [47], macacos coronados [48] y papiones jóvenes [55].
Nivel de testosterona	Topillos agrestes [33], bovinos [56] y humanos [57].		
Momento de inseminación	Hámsteres [62], ciervos de cola blanca [63], ovinos [64] y bovinos [65, 66].	Conejos [74], cerdos [75], bovinos [61, 76, 77] y humanos [9].	Ratones [67], bovinos [68] y humanos [69, 70].
Momento de inseminación (forma de U)	U invertida: Bovinos [68] y humanos [71].		U: Ratas [72] y humanos [71, 73].
Lado de ovulación/gestación	Machos en la derecha: Gerbillos de Mongolia [80] y bovinos [78, 79].	Ratones [83], ratas [84] y cerdos [85]	Machos en la izquierda: Conejos [81] y ratones [82]
Estrés	Crónico: Marsupiales [98], primates [99] y humanos [100, 101].	Corticosterona: Topillos agrestes [33].	Agudo: Ratas [88, 90], hámsteres [89, 91] y humanos [92-97].
Edad	Topillos agrestes [33] y bovinos [109]. Forma de U invertida: Ovinos [50].	Monos rhesus [60].	Ciervos de cola blanca [107] y humanos [110-112]. Forma de U: Hámsteres [108].
Número de parto	Forma de U invertida: Hámsteres [108].	Bovinos [76, 115] y monos rhesus [60].	Humanos [113, 114].
Tamaño de camada	Topillos agrestes [33].	Cerdos [75].	Cobayas [116] y jabalíes [117].
Condiciones ambientales químicas o físicas	Clima cálido: Bovinos [109, 115] y humanos [140].		Contaminantes: Humanos [119-122]. Cafeína: hámsteres chinos [128]. Alto voltaje: Humanos [129].
Tabaco (humanos)	[123]	[125, 126]	[124]
Condiciones médicas adversas (humanos)	Hepatitis B [136], cáncer de pecho [137] y tras la infección por *Toxoplasma gondii* en humanos [138] y ratones [139].		Linfoma no-Hodgkin [131], hepatitis [132], tratamiento con clomifeno [132], hiperplasia adrenal congénita [133] e infección por *Cytomegalovirus* [134, 135].

Sesgos de la proporción de sexos originados en el macho

La mayoría de los estudios de la proporción de sexos secundaria se basan en un supuesto efecto materno, pero algunos artículos han descrito un efecto paterno sobre la proporción de sexos. Dejando a un lado las enfermedades ligadas al cromosoma X, que dan lugar a un aumento de la proporción de sexos debido a la mortalidad femenina, es difícil aportar un mecanismo biológico que explique un sesgo en la proporción de sexos de base paterna, dado que el macho produce la misma cantidad de espermatozoides X e Y [143, 144]. Por ello se ha sugerido que los supuestos efectos paternos en la proporción de sexos secundaria en humanos son el resultado de efectos de segundo orden debido al emparejamiento selectivo [46].

En humanos se ha encontrado una tendencia hacia una correlación negativa entre la edad paterna y la proporción de sexos [113, 114, 145]. Sin embargo, el porcentaje de espermatozoides Y no se vió afectado por la edad [146] y por ello, las diferencias pueden ser causadas por una interacción entre la edad materna y el número de parto y la edad paterna. Además, se ha propuesto que en vez de la edad de cada progenitor, la diferencia de edad entre ambos puede predecir el sexo del primer hijo: los padres con una diferencia de edad mayor obtuvieron un exceso de niños en su descendencia, y la situación opuesta se observó en las parejas con una edad menor [147].

En ciervos europeos (*Cervus elaphus*) se ha descrito una correlación positiva entre la fertilidad del macho y el porcentaje de descendencia macho [148]. El estudio eliminó la interacción de factores de la hembra realizando inseminación artificial y así, los autores propusieron que el efecto podía ser causado por una diferencia en la proporción de espermatozoides X o Y en el eyaculado o por una supuesta ventaja en la fecundación de los espermatozoides Y comparados con los X cuando fueran producidos por los machos más fértiles, mientras que la situación opuesta podría ocurrir en los machos menos fértiles. La primera hipótesis se apoya en el descubrimiento de variaciones en la proporción de sexos entre eyaculados del mismo toro [149], aunque estos resultados no se han repetido. La segunda se puede explicar mediante la presencia de delecciones del cromosoma Y en los machos con baja fertilidad, ya que en ratones se ha observado que las delecciones en el cromosoma Y

producen espermatozoides Y con anomalías morfológicas que son menos eficaces en la fecundación dando lugar a bajas proporciones de sexos [150]. De acuerdo con el nexo entre fertilidad y proporción de sexos, los hombres con condiciones médicas desfavorables tales como cáncer testicular [151, 152], o expuestos a sustancias nocivas como las dioxinas [121], el ditiotreitol [153], el dibromocloropropano [154], fungicidas [155], el boro [156], residuos de aluminio [157], o el tabaco [124, 158] experimentan un sesgo a favor de las hembras en la proporción de sexos de su descendencia que se ha relacionado con bajos niveles de testosterona paterna [159], mientras que los hombres tratados con una terapia de metiltestosterona engendraron más hijos que hijas (45:17) [160]. Siguiendo la misma tendencia, la administración experimental de dioxina al macho antes de la monta disminuyó la proporción de sexos en ratones [161] y cobayas [162]. Sin embargo, se han observado que las reducciones leves en la fertilidad no alteran la proporción de sexos en el hombre [163]. En la especie bovina, se ha sugerido que la frecuencia de extracción del semen puede tener un efecto sobre la proporción de sexos [164]. Por último, en un ámbito similar a los efectos paternos, se ha descrito que la inseminación artificial puede aumentar la probabilidad de obtener terneros en vacuno lechero y cárnico, aunque sólo se detectó un incremento del 1 % [109]. El mismo estudio también indicó que las razas cárnicas sesgaban la proporción de sexos a favor de los machos [109].

Mecanismos biológicos de la influencia parental sobre la proporción de sexos

La mayoría de los sesgos en la proporción de sexos que han sido citados en estas líneas se basan en estudios retrospectivos, que adolecen del apoyo de un mecanismo biológico para ser completamente fiables. Las complejas relaciones entre las diferentes variables que pueden influir en el sexo de la descendencia (ej. condición materna, dominancia, glucosa, testosterona, momento de inseminación…) hacen que las observaciones y las hipótesis derivadas de ellas sean bastante especulativas sin una base molecular o fisiológica. De hecho, se ha sugerido que algunas de las alteraciones de la proporción de sexos publicadas no son reales, sino causadas únicamente por el azar [86]. Debido a su importancia y a la sencilla toma de datos, la proporción de sexos es un parámetro analizado con frecuencia, que no se suele obviar en cualquier tipo de análisis de una población animal. De este modo, cientos de análisis de la proporción de sexos son llevados a cabo cada año en una diversidad de estudios que

implican distintas especies y parámetros. Entre tal número de observaciones, algunas obtendrán diferencias significativas, y éstas serán las únicas que serán publicadas. Por estas razones, para determinar el verdadero significado de las distintas observaciones son necesarios los estudios basados en los mecanismos, junto con los metaanálisis y los marcadores fisiológicos.

Cuando se ha analizado la consistencia y la coherencia de los diferentes artículos publicados mediante metaanálisis, se suele llegar a la conclusión de que el efecto puede ser debido a la variación estocástica en muestras pequeñas [58]. Sin embargo, una revisión restringida a ungulados encontró un apoyo consistente a la relación entre dominancia y condición materna y proporción de sexos de la descendencia, y que las medidas de la condición materna tomadas en torno a la concepción proporcionan el mayor apoyo a la hipótesis de Trivers y Willard [165]. De acuerdo con esto, otro metaanálisis basado en estudios en mamíferos excluyendo a la especie humana, determinó que el ajuste de la proporción de sexo ocurre en o cerca de la implantación [42]. Además, en las especies domésticas, el comienzo de la lactación, el periodo de mayor demanda energética, coincide en el tiempo con la concepción, y por ello este periodo es el más adecuado para establecer una relación entre condición corporal y la futura capacidad inversora de energía de la madre.

Para explicar las diferencias entre estudios, se han propuesto dos marcadores fisiológicos. En primer lugar, la concentración materna de glucosa alrededor de la concepción puede unir distintos resultados [42], ya que es alta en madres en una buena condición, pero también aumenta bajo estrés social, es alta en diabetes y varía en condiciones médicas adversas, varía a lo largo del ciclo y está relacionada con la LH [166, 167]. En segundo lugar, los niveles maternos de testosterona pueden explicar los resultados contradictorios relacionados con la condición materna, el nivel de dominancia y el estrés [14] –ya que el estrés crónico altera los niveles de testosterona disminuyéndolos en machos y aumentándolos en hembras-, y las alteraciones de la proporción de sexos causadas por diferentes condiciones médicas adversas y contaminantes [168].

Los mecanismos responsables de la alteración de la proporción de sexos pueden ocurrir en dos periodos distintos: antes de la fecundación o después de la

misma mediante la pérdida selectiva de embriones/fetos de un sexo determinado. En la mayoría de los estudios descriptivos es difícil establecer si las proporciones de sexo atípicas se originaron en la concepción, durante la gestación o en ambos periodos, puesto que los efectos ambientales están presentes antes y después de la concepción [14]. Sin embargo, desde un punto de vista evolutivo, los mecanismos que actúan tempranamente (antes o en torno a la concepción) constituyen una ventaja con respecto a los que actúan más tarde, ya que los últimos representan un mayor derroche al suponer la pérdida de embriones o fetos, con una consiguiente pérdida de fertilidad.

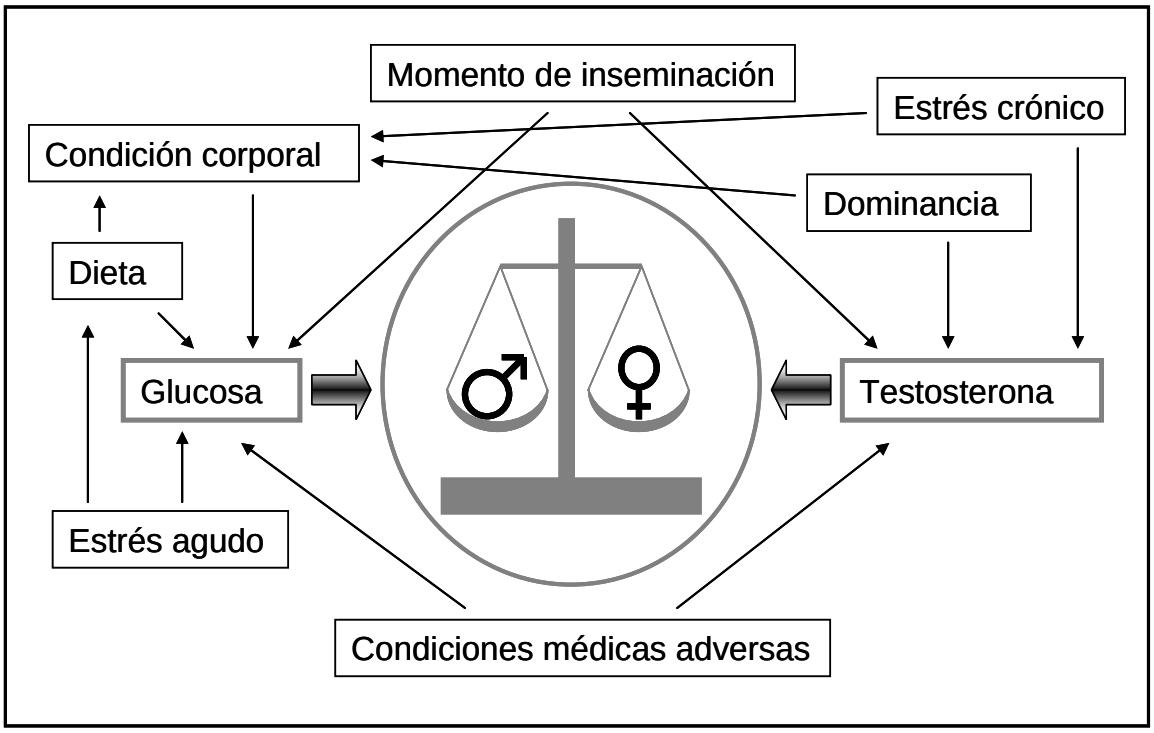


Figura 1: Relaciones entre las distintas características maternas que pueden alterar la proporción de sexos y los dos marcadores fisiológicos propuestos para explicar las discrepancias entre estudios.

Mecanismos preconcepcionales

Antes de la concepción, los mecanismos de control de la proporción de sexos deben implicar al ovocito o al espermatozoide. En el primer caso, se requeriría de un supuesto mecanismo de selección espermática llevado a cabo en el lugar de la fecundación mediante las células del cúmulo o la zona pelúcida, mientras que el segundo puede producirse por diferencias en motilidad, viabilidad, reacción acrosómica, mortalidad o interacción con el tracto genital femenino, entre

espermatozoides X o Y, que conducirían a una diferencia en la probabilidad de alcanzar el lugar de la fecundación.

La hipótesis de una supuesta selección preferente de espermatozoides X o Y fue formulada por primera vez por Dominko y First [169] y require de la presencia de proteínas específicas del sexo en la membrana del espermatozoide que permitirían que el complejo cúmulo-ovocito los pudiese distinguir. La existencia de estas proteínas específicas de sexo es teóricamente posible gracias a la transcripción postmeiótica [170-174]. En base a esta hipótesis, se ha publicado que el estado de maduración del ovocito madurado *in vitro* ejerce una influencia sobre la proporción de sexos en la especie bovina [64, 169, 175]. En particular, se encontró una correlación positiva entre el tiempo de maduración y la proporción de sexo, lo que aporta un mecanismo para explicar el aumento de la proporción de sexo observado en inseminaciones tardías [64-66]. Este asunto particular será tratado en el capítulo 1. En la misma línea, el nivel de testosterona del fluido folicular se ha correlacionado de forma positiva con la probabilidad de que el ovocito sea fecundado *in vitro* por un espermatozoide Y [176], lo que concuerda con el aumento de la proporción de sexos observado en las madres dominantes [54, 56]. La posibilidad de una selección del sexo llevada a cabo por el ovocito tiene grandes repercusiones en el uso de semen sexado en la producción animal, ya que si los ovocitos están predeterminados a ser fecundados por un espermatozoide X o Y, las tasas de fertilidad serán bajas a pesar del avance en las técnicas de separación espermática [177]. Esta hipótesis será probada en el capítulo 2.

Se ha propuesto que las diferencias en motilidad [178], migración en mucus cervical [179], tasa de capacitación [65], y supervivencia [180] entre espermatozoides X e Y pueden influir en la proporción de sexos. En base a estas diferencias, se han desarrollado varios métodos de separación del esperma, pero ninguno produce resultados consistentes, y el empleo de semen sexado por citómetro del flujo sigue siendo el único método fiable, como será explicado posteriormente. Se ha sugerido que en humanos los espermatozoides Y son más mótiles que los X [178], aunque un estudio posterior, que analizó el esperma X y el Y, concluyó que el esperma Y de toro no nada más rápido que el X en soluciones salinas simples, aunque mostró mayor linearidad y rectitud de ruta que el X [181]. De acuerdo con una mayor motilidad de los espermatozoides Y, Kochhar y Kochhar encontraron que reduciendo el tiempo de

coincubación (6 h) de la FIV en la especie bovina, disminuía la tasa de división y aumentaba la proporción de sexos [182]. Kiwata et al. obtuvieron resultados similares, aunque el sesgo de la proporción de sexos fue menor [183]. Ambos estudios no encontraron una variación en la proporción de sexos cuando se prolongó la coincubación, lo que indica que los espermatozoides X sobreviven más tiempo. De acuerdo con esto, la preincubación del esperma bovino durante 24 horas antes de la FIV disminuyó la proporción de sexos [180]. En oposición, se ha descrito en un estudio *in vitro* que la supervivencia de los espermatozoides Y humanos es ligeramente mayor [184].

La ausencia de diferencias de motilidad en soluciones salinas simples [181] no excluye la posibilidad de que puedan surgir en condiciones fisiológicas. Se ha propuesto que la conductividad de las secreciones vaginales de la vaca puede influir en la proporción de sexos [65]. Los autores describieron una fuerte relación entre la conductividad, la proporción de sexos y el momento de la aparición del estro: al inicio del estro (20 horas antes de la ovulación) la conductividad fue baja y se obtuvieron más terneras (92,9 %), mientras que después (10 horas antes de la ovulación), la conductividad aumentó y se obtuvieron más terneros (91,7 %). Los autores argumentaron que los espermatozoides Y capacitaban antes que los X. Así, en inseminaciones tempranas, los espermatozoides Y capacitan antes y pierden la capacidad fecundante antes de alcanzar al ovocito, mientras que en inseminaciones tardías la capacitación temprana suponía una ventaja para fecundar [65]. Desafortunadamente, aunque se ha descrito una relación inversa entre la resistencia (conductividad) de las secreciones vaginales y la concentración de estrógenos [185], el sesgo extremo de la proporción de sexos no se ha confirmado en otros estudios. En la misma línea, en humanos se ha propuesto un posible nexo entre la proporción de sexos y las variaciones de estado del mucus cervical causadas por los cambios en el ambiente hormonal antes de la concepción [186]. A consecuencia del aumento en la producción de estradiol, la penetrabilidad del mucus cervical tiende a aumentar durante los 6 días previos a la ovulación, mientras que el aumento de la producción de progesterona por el cuerpo lúteo después de la ovulación la disminuye rápidamente. Las inseminaciones tempranas no fecundantes también disminuyen la penetrabilidad, porque ensucian los canales del mucus con espermatozoides, leucocitos y otros detritos. Asumiendo esta máxima y que los espermatozoides Y pueden atravesar más

fácilmente el os cervical, el autor propuso que la proporción de sexos en la concepción estaba correlacionada positivamente con la frecuencia coital y no variaba con el momento de la inseminación fecundante cuando la frecuencia coital era alta [186]. En la misma línea, el bicarbonato actúa en la capacitación espermática y en la interacción espermatozoide-ovocito [187] y, por ello, el aumento en la proporción de sexos asociado a montas tardías observado en hámsteres se explicó mediante un descenso del pH vaginal [62].

Selección del sexo del esperma

Aunque existen más de 100 patentes que aseguran ser capaces de sexar esperma satisfactoriamente, la mayoría de los procedimientos no son más eficaces que los métodos tradicionales con más de dos milenios de antigüedad [188]. En la actualidad, el sexado por citometría de flujo es el único método fiable, dado el fracaso de otros métodos [189, 190].

Los espermatozoides X e Y se diferencian en peso y cantidad de ADN, permitiendo su separación por citometría de flujo. Los intentos iniciales de separación espermática se basaron en una diferencia teórica en la carga eléctrica, debida a diferencias en el contenido de ADN o a unas hipotéticas diferencias en la membrana. Sin embargo, en bovino, la separación de esperma por electroforesis [191] o los geles newtonianos insolubles en agua [192] no consiguieron modificar la proporción de sexos. Las diferencias en contenido de ADN provocan una diferencia en el peso del espermatozoide que ha inspirado el desarrollo de métodos basados en la sedimentación o gradientes de densidad, tales como el *Percoll*, la albúmina, el *Sephadex* o la lana de vidrio. Se publicó que ciertos gradientes de *Percoll* alteraban la proporción de sexos en humanos [193-195] y causaban un ligero cambio en bovinos [196], pero otros estudios en humanos [197, 198] o bovinos [199-201] no pudieron repetir estos resultados. También se ha indicado que cierto gradiente de albúmina seleccionaba los espermatozoides Y en bovinos [202] y humanos [203], aunque otros grupos contradicen estos resultados en conejos [74], bovinos [144, 204] y humanos [143, 205-209], e incluso se ha obtenido un descenso en la proporción de sexos en humanos [210]. Las columnas de *Sephadex* [208, 211] y la lana de vidrio [198] en

humanos y los métodos de sedimentación en conejos [212, 213] también han fracasado en el intento de alterar la proporción de sexos.

Los métodos basados en las supuestas diferencias en motilidad, susceptibilidad al pH y proteínas de membrana han conducido a resultados negativos o inconsistentes. En la especie bovina, el *Swim-up* doble aumentó el porcentaje de espermatozoides Y [214], pero no sirvió para seleccionar el sexo en humanos [195, 198, 215-217]. En conejos, el cambio de pH del plasma seminal no alteró la proporción de sexos [218]. El sexaje inmunológico del esperma, basado en las hipotéticas proteínas específicas de sexo presentes en el espermatozoide, obtuvo éxitos parciales [219] y aunque se anunció su utilidad en bovinos [220], otros grupos no pudieron detectar los antígenos específicos de sexo [171, 221, 222].

La separación del esperma por citometría de flujo se basa en las diferencias en su contenido en ADN, medidas de forma indirecta en base a la fluorescencia emitida por la tinción de ADN Hoetchst 33342. Desde la primera publicación de sexado por citometría de flujo [223, 224] y la primera cría obtenida con semen sexado [225, 226], los citómetros/separadores de flujo han evolucionado, sobre todo en términos de velocidad de separación [188, 227], y en la actualidad hay una amplia disponibilidad comercial de semen sexado bovino. A pesar de que Reino Unido fue el primer país en comercializar de forma eficaz el semen sexado [11], actualmente es producido y usado a mayor escala en Estados Unidos debido a razones comerciales y productivas, como la mayor demanda de novillas de reemplazo en comparación con Europa [228]. En Estados Unidos, las inseminaciones con semen sexado en novillas correspondieron a un 1,5, 9,6 y 14,2 % del total de las cubriciones en los años 2006, 2007, and 2008, respectivamente, mientras que en vacas, supusieron un 0,1, 1,3 y 2,1 %, lo que muestra un incremento en su uso [229]. Aunque la exactitud del sexaje por citometría de flujo oscila en torno al 90 %, en la mayoría de las especies, incluyendo el ganado vacuno [228, 230, 231], la tecnología es bastante costosa para el ganadero y el centro de inseminación. El coste anual de funcionamiento de una instalación de semen sexado puede exceder los 2 millones de dólares estadounidenses [11], y con respecto al ganadero, además del elevado coste de la dosis seminal, el uso de semen sexado en condiciones de granja normalmente conduce a una reducción de la fertilidad (revisado en [232]). Los tres factores que ejercen una mayor influencia en la rentabilidad del

uso del semen sexado son la diferencia de valor entre la descendencia macho y hembra, y las diferencias en precio y fertilidad entre las dosis de semen sexado y sin sexar. Sin embargo, es difícil tener en cuenta todos los factores que podrían incidir en el beneficio económico del uso del semen sexado tales como la exactitud del sexaje (que normalmente ronda el 90 %), el coste del fallo en la fecundación, las ventajas de la reducción de la incidencia de distocias en las novillas preñadas con una ternera en vez de un ternero [233], el aumento en la producción lactea motivado por el menor peso del feto hembra [234], la ganancia genética obtenida con el semen sexado [235], o las oportunidades de explotar empresas y estrategias alternativas [11]. Después de asumir una serie de supuestos, se llegó a la conclusión de que los terneros del sexo más preciado deberían valer al menos 200 dólares estadounidenses más que el sexo de menor valor para que el uso de semen sexado tuviera sentido desde un punto de vista económico [11].

Entre los diferentes factores que afectan al rendimiento económico del uso de semen sexado, la disminución de la fertilidad provoca pérdidas considerables [11]. Usando semen sexado, las tasas de concepción en el primer servicio promedian en Estados Unidos un 47 % en novillas Holstein y un 53 % en novillas Jersey, siendo en torno a un 80 % de las logradas con semen convencional [230]. En un sistema de producción europeo, concretamente en Dinamarca, la tasa de concepción del semen sexado se redujo con respecto al empleo de la dosis convencional en un 5 % en Roja Danesa (60.2 %), un 7 % en Jersey (46.6 %) y un 12 % en Holstein (49.3 %) [231]. En Holanda, Frijters et al. obtuvieron resultados similares, con un descenso del 13,6 % en las tasas de no retorno en Holstein, y atribuyeron dos tercios (8,6 %) a la baja dosis y uno (5 %) al procedimiento de sexaje [236]. Para explicar la reducción en las tasas de fertilidad, se han propuesto varios factores, incluyendo la baja dosis de espermatozoides usada [237], el daño espermático causado por el procedimiento de separación [238] y una supuesta selección espermática llevada a cabo por el ovocito [176]. Estas hipótesis serán abordadas en los capítulos 2 y 3.

Aunque se ha obtenido descendencia normal tras el empleo de semen sexado (revisado en [239]), se ha observado que los espermatozoides dañados pueden fecundar ovocitos dando lugar a embriones de baja calidad [240]. En el ámbito de esta hipótesis, dos publicaciones han encontrado anomalías en la expresión génica [241] o

ultraestructura [242] en embriones producidos con semen sexado, que pueden desembocar en mortalidad embrionaria. En función de los datos de granja disponibles, cualquier aumento en la mortalidad embrionaria causado por el uso de semen sexado parece ocurrir tan temprano que no afecta al periodo de reconocimiento maternal y, de este modo, un alto porcentaje de hembras vuelven al celo en un intervalo interestral normal [230], lo que también es compatible con la ausencia de fecundación. Sin embargo, los embriones de baja calidad pueden llegar a término dando lugar a efectos a largo plazo en la descendencia [243]. El posible efecto del semen sexado sobre la calidad del embrión, evaluado en base a la expresión génica y velocidad de desarrollo será analizado en el capítulo 3.

A pesar de que la tecnología de semen sexado se emplea principalmente en el ganado vacuno, la misma se ha aplicado a una diversidad de especies, incluyendo al conejo [226], al gato [244], al perro [245], al cerdo [246], al caballo [247], al morueco [248], a los primates no humanos [249], y al hombre [250]. En humanos, el uso de semen sexado como medio de selección del sexo constituye una alternativa a la biopsia embrionaria, evitando los posibles efectos de la manipulación de embriones y la destrucción de los embriones del sexo no deseado. Por ello, existe un interés creciente en el empleo de semen sexado como método de selección preconcepcional que podría ser empleado para disminuir el riesgo de enfermedades ligadas al sexo o para equilibrar la distribución de hijos en una familia [251]. La tecnología del semen sexado también es de utilidad para la conservación y manejo de animales salvajes [252].

Mecanismos postconcepcionales

La proporción de sexos se puede modificar mediante la pérdida selectiva de embriones o fetos de un sexo determinado. Estos mecanismos, al contrario que los preconcepcionales, implican una pérdida de fertilidad y éxito reproductivo y, por tanto, es más probable que ocurran en especies politocas con una descendencia numerosa, en la que cada individuo tiene unas probabilidades relativamente bajas de sobrevivir hasta la edad adulta (estrategas de la r), que en especies que invierten más en una descendencia menos numerosa (estrategas de la k). En el caso de los estrategas de la r, la pérdida selectiva puede ocurrir incluso después del nacimiento, como

sugirieron Trivers y Willard [15], y ha sido confirmado en algunos roedores [83, 253, 254].

Entre los distintos sesgos de la proporción de sexos descritos, los mecanismos postconcepcionales pueden explicar aquellos que van acompañados de una reducción en la fertilidad, especialmente aquellos observados bajo condiciones adversas o estresantes. Estas variaciones de la proporción de sexos bajo condiciones adversas o estresantes pueden ocurrir en estrategas de la r o o de la k. Aunque en condiciones *in vitro* se ha observado una pérdida preferente de embriones hembra en bovinos [255], se piensa que los embriones y fetos macho son más sensibles a condiciones adversas que los hembra. En humanos, hay una mayor pérdida de fetos macho que de hembras [256]. De acuerdo con las bajas proporciones de sexo encontradas en casos de exposición materna a contaminantes, varios estudios han encontrado una mayor susceptibilidad de los fetos macho comparados con las hembras a contaminantes ambientales como los pesticidas [257, 258]. De forma similar, se ha visto que el hábito de fumar de la madre retrasa el crecimiento fetal de los machos en mayor medida que el de las hembras [259], aunque el efecto antiestrogénico del tabaco también proporciona un nexo entre el consumo de tabaco, los niveles bajos de testosterona y las bajas proporciones de sexos [260]. En el caso de las variaciones de la proporción de sexos causadas por estrés, también se ha propuesto una mayor mortalidad de los fetos macho en comparación con las hembras [261], y se ha observado en ratas [262] y hámsteres [91], que ciertos sesgos de la proporción de sexos a favor de las hembras ocurren debido a una pérdida preferente de fetos macho.

Cuanto antes ocurra la pérdida selectiva de embriones o fetos, menos se verán afectados el éxito reproductivo y las reservas energéticas maternas. Cuando la pérdida selectiva acontece de forma temprana en el periodo preimplantacional, antes del reconocimiento materno, la hembra vuelve al estro en un intervalo interestral normal y su pérdida energética es comparable a una inseminación no fecundante. La pérdida selectiva durante estos estadios tempranos implica la existencia de un dimorfismo sexual que ocurre antes de la diferenciación gonadal, hecho que, como se explicará más adelante, ha sido demostrado. Se ha descrito que el metabolismo total de glucosa es dos veces más alto en machos que en hembras, y que la actividad de la ruta de las pentosas fosfato es cuatro veces mayor en blastocistos bovinos hembra que en machos

[263]. También se ha observado un mayor consumo de piruvato y glucosa en embriones macho humanos [264]. En esta línea, se ha observado una relación positiva entre la concentración de glucosa y la proporción de sexos en el cultivo *in vitro* bovino [265] y que la glucosa acelera el desarrollo de los embriones macho y retrasa el de las hembras [266, 267]. Los estudios transcripcionales han mostrado una mayor expresión del gen ligado al cromosoma X glucosa-6-fosfato deshidrogenasa (*G6PD*) en bovinos [268-270] y humanos [271], que puede causar las diferencias metabólicas descritas. G6PD es la primera enzima regulatoria clave de la ruta de las pentosas fosfato, siendo esencial para el aporte del poder reductor de NADPH y constituyendo un nexo entre la respuesta al estrés y el dimorfismo sexual transcripcional que puede explicar por qué los embriones hembra sobreviven mejor a las condiciones de estrés oxidativo en ratones [272]. En este sentido, se ha demostrado que la inhibición de G6PD anula parte del dimorfismo sexual en ratones [272] y bovinos [265]. Además, se ha propuesto a G6PD como candidato para explicar las variaciones en la proporción de sexos causadas por la dieta (revisado en [266]). En una aproximación *in vivo* para determinar el papel de la glucosa en el control de la proporción de sexos, se encontró un descenso de la proporción de sexo y los niveles de glucosa en ratones tratados con dexametasona [273]. De forma similar, se ha observado que la suplementación de glucosamina en el medio de cultivo después del estadio de 8 células disminuye las tasas de desarrollo embrionario y sesga la proporción de sexos a favor de los machos [274]. Además, el efecto fue neutralizado al añadir un inhibidor de la enzima O-N-acetilglucosamina transferasa (OGT), codificada en el cromosoma X e implicada en el metabolismo de la glucosamina [274].

Dimorfismo sexual preimplantacional

El dimorfismo sexual preimplantacional constituye una base fisiológica para las diferencias en la tasa de supervivencia entre embriones de distinto sexo observadas bajo condiciones adversas. Sin embargo, esta no es la única repercusión del dimorfismo sexual preimplantacional. El estudio de este fenómeno en los estadios iniciales del desarrollo ayuda a la comprensión de fenómenos como la regulación transcripcional de los cromosomas sexuales, la inactivación temprana del cromosoma X –que sólo ha sido estudiada al detalle en ratones-, la determinación temprana del sexo, la evolución de los cromosomas sexuales, las enfermedades ligadas al

cromosoma X, y los efectos a largo plazo específicos del sexo relacionados con el origen embrionario de la enfermedad adulta.

Los estudios iniciales sostenían la idea de que los embriones de ambos sexos se desarrollaban de forma similar hasta el desarrollo de las gónadas [275]. Sin embargo, ahora se sabe que los embriones preimplantacionales exhiben un dimorfismo sexual antes de que ocurra la diferenciación gonadal, que sólo puede atribuirse a sus diferencias en la dosis de cromosomas sexuales [276]. Además de las diferencias en el metabolismo de glucosa ya descritas, en mamíferos euterios se ha sugerido que los embriones macho sobreviven mejor a la vitrificación [277], y se desarrollan más rápido que sus compañeras antes de que ocurra la diferenciación sexual. Así, se han encontrado diferencias entre sexos en velocidad de desarrollo en ratones [278], suidos [279], ovinos [280], bovinos [255, 281-284], y humanos [285]. Si esta idea es cierta, se esperaría un sesgo en la proporción de sexo al utilizar técnicas *in vitro*, ya que tanto en humanos como en animales de granja, se considera que embriones más rápidos son de mayor calidad, y por ello son seleccionados para ser transferidos. Sin embargo, en humanos no se ha observado un sesgo de la proporción de sexos tras transferir embriones de rápido desarrollo [286, 287]. En la misma linea, no se han visto diferencias entre sexos en la velocidad de desarrollo en ratones [16, 288], ratas [84], suidos [289, 290], ovinos [43] y bovinos [288, 291]. Las condiciones de cultivo *in vitro* pueden ser las causantes de las diferencias en velocidad de desarrollo que pueden llevar a una pérdida o selección de embriones de un sexo determinado. De este modo, en ratones se ha indicado que la aceleración en el desarrollo preimplantacional de los machos ocurre *in vitro*, pero no *in vivo* [292]. También en bovinos, se obtuvo un aumento en la proporción de sexos entre los blastocistos expandidos entre los embriones cultivados *in vitro*, pero no en los cultivados en el oviducto ovino [293], y, como se ha comentado anteriormente, la presencia de una alta concentración de glucosa originó diferencias entre sexos en velocidad de desarrollo que no se observan en niveles óptimos [267]. Tampoco se obtuvieron diferencias en embriones bovinos cultivados sin suero [294]. En humanos, la transferencia de embriones de estadios tempranos no alteró la proporción de sexos [295], pero hay varios artículos que muestran un sesgo de la proporción de sexos cuando se transfieren embriones en estadio de blastocisto [296-298]. En la misma tendencia, se ha publicado que sólo los embriones macho obtenidos por ICSI, pero no

aquellos obtenidos por FIV, crecen más rápido que sus compañeras [299]. De acuerdo con la supuesta mayor velocidad de desarrollo, se ha visto que los embriones macho producidos *in vitro* contienen más células que las hembras en bovinos [255] y humanos [285]. También en humanos, los fetos macho del tercer trimestre son más grandes que las hembras, lo que se atribuyó a diferencias en la constitución cromosómica [300]. Sin embargo, otros estudios no han encontrado diferencias entre sexos en bovinos [284, 301]. El dimorfismo sexual preimplantacional en términos de diferencia de supervivencia en el cultivo *in vitro* y velocidad de desarrollo será estudiado en los capítulos 1 y 3.

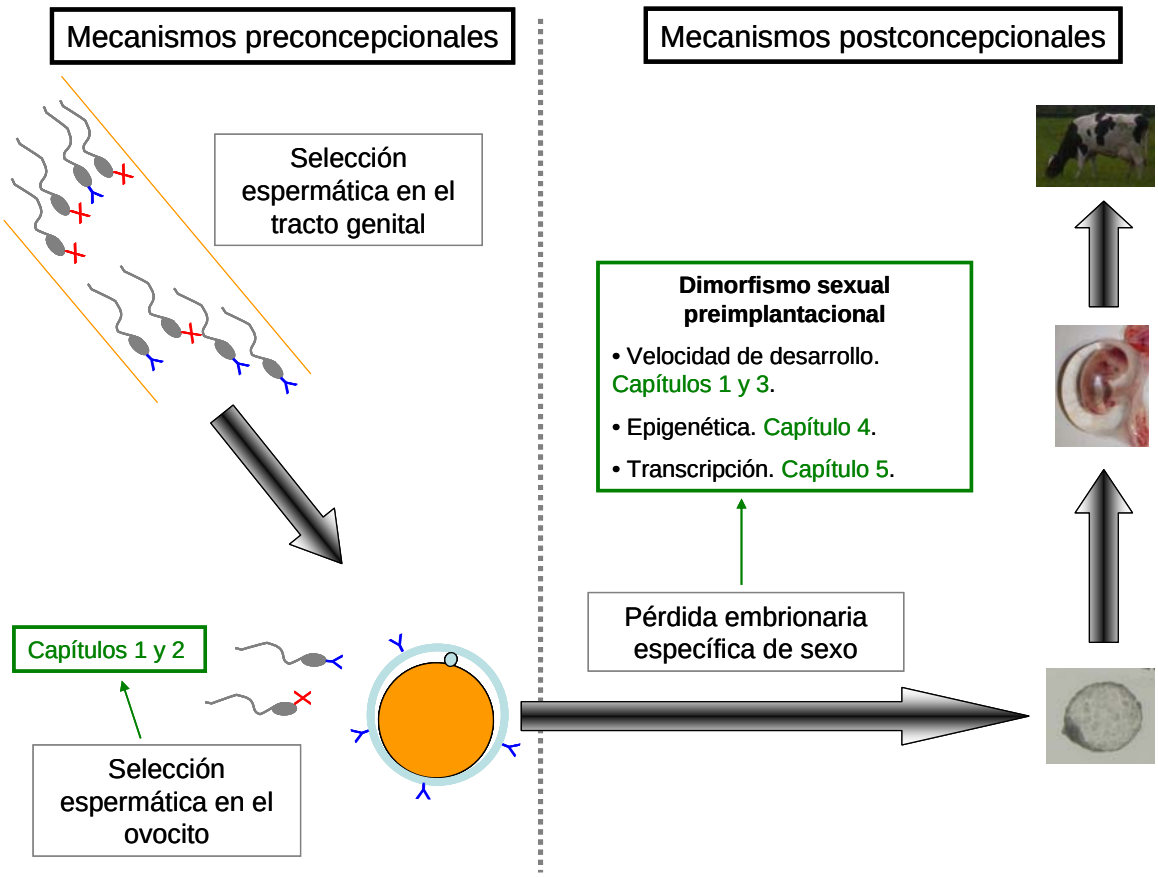
El dimorfismo sexual preimplantacional es la consecuencia de un dimorfismo transcripcional causado por las diferencias en la dosis de cromosomas sexuales. Los genes ligados al Y se expresan exclusivamente en el macho y por ello, la primera descripción de diferencias transcripcionales entre sexos en embriones preimplantacionales fue el antígeno de histocompatibilidad Y, que estaba presente en el 50 % de los embriones de ratón de 8 células [302], que posteriormente se confirmó que eran machos [303]. Después, se descubrió la transcripción de SRY en embriones macho de ratón [304, 305], bovinos [306] y humanos [307]. Los genes del cromosoma X están presentes en una dosis doble en hembras, pero en los tejidos adultos se consigue un nivel transcripcional igual para ambos sexos gracias a la compensación de la dosis mediante la inactivación aleatoria del cromosoma X. Sin embargo, durante el periodo preimplantacional, la inactivación del X es un proceso reversible y dinámico [308] y algunos genes escapan a la inactivación y se expresan en un mayor nivel en embriones macho, como ha sido descrito en embriones de ratón [309-312], bovinos [241, 268, 269, 313] y humanos [271]. La extensión de este escape de la inactivación es desconocida. Además de este efecto directo sobre la expresión de genes, los genes localizados en los cromosomas sexuales modulan la expresión de los genes autosómicos, dando lugar a diferencias entre sexos [241, 314]. Entre los escasos genes autosómicos de los que se conoce un dimorfismo sexual en sus patrones de expresión, el interferón Tau (IFNT) –que informa de la gestación a la madre en los rumiantes [315]- ha sido ampliamente estudiado. Los blastocistos bovinos hembra produjeron más IFNT que los machos en condiciones *in vitro* [314] e *in vivo* [316], aunque las diferencias desaparecieron en embriones elongados de día 14 [316]. De forma similar, los embriones hembra ovinos de día 13 produjeron una cantidad

ligeramente mayor de IFNT que los macho [43] y en ciervos europeos (*Cervus elaphus*) el IFNT fue detectado en el 60 % de 10 blastocistos hembra y no se detectó en ninguno de los 7 blastocistos macho analizados [19]. Estas diferencias pueden estar unidas al metabolismo de glucosa, ya que la inhibición de G6PD en embriones bovinos, suprimió la expresión diferencial de *IFNT* [317]. El dimorfismo sexual en la transcripción de genes candidatos será abordado en los capítulos 3 y 4. La mayoría de los estudios que han analizado el dimorfismo sexual transcripcional preimplantacional se basan en un limitado número de genes candidatos. Los estudios de expresión génica global proporcionan una estimación de la extensión del dimorfismo sexual transcripcional que ayuda al conocimiento de fenómenos como la regulación transcripcional de los cromosomas sexuales, la inactivación del cromosoma X y los sesgos de la proporción de sexos debidos a mecanismos postconcepcionales. Sin embargo, debido a las dificultades técnicas para la obtención de la gran cantidad de embriones por grupo necesarios para llevar a cabo las técnicas de biología molecular, sólo una publicación, basada en ratones, ha analizado las diferencias entre sexos en la expresión génica global [318]. En el capítulo 5, se analizarán las diferencias entre sexos en la expresión génica global en el modelo bovino.

En biología, el término epigenética se refiere a características como las modificaciones de la cromatina y del ADN que son estables después de varios ciclos de división celular pero no implican cambios en la secuencia de ADN del organismo [319]. La epigenética controla la transcripción del ADN y constituye la vía por la cual el genoma integra las señales intrínsecas y ambientales [320]. El estatus epigenético, especialmente los niveles de metilación de ADN o de metilación o acetilación de histonas, es la base de las diferencias transcripcionales y, por ello, el dimorfismo sexual transcripcional debe iniciarse mediante fenómenos epigenéticos. Sin embargo, durante el desarrollo preimplantacional, la única diferencia epigenética descrita entre sexos es la inactivación del cromosoma X. Aparte de las modificaciones en el ADN o las histonas, hay otras modificaciones epigenéticas, como el número de copias de ADN mitocondrial y la longitud telomérica. Las mitocondrias son las responsables de la glucolisis oxidativa, cuya importancia como una fuente significativa en la producción de ATP aumenta durante el periodo preimplantacional [321-323], coincidiendo con un aumento en la maduración mitocondrial [324] y en su número [325]. Debido a su importancia en dos factores implicados en el dimorfismo sexual

preimplantacional (metabolismo del embrión y estrés oxidativo [272]), se ha propuesto que las mitocondrias pueden tener un papel importante en las diferencias entre sexos [326]. Los telómeros son secuencias de ADN repetidas situadas en el extremo de los cromosomas cuya longitud determina el límite de divisiones que puede llevar a cabo una célula somática adulta antes de la senescencia [327]. La longitud telomérica queda determinada en el periodo preimplantacional, cuando los telómeros se elongan mediante un mecanismo alternativo basado en recombinación [328] que se continúa con la telomerasa [329]. En el capítulo 5, se determinarán las diferencias epigenéticas entre sexos en el estadio de blastocisto en términos de expresión génica de enzimas relacionadas con la epigenética, en el estatus de metilación de secuencias repetidas, en el número de copias de ADN mitocondrial y en la longitud telomérica.

Figura 2: Posibles mecanismos de control de la proporción de sexos y sus relaciones con los capítulos de esta tesis.



Bibliography/Bibliografía

1. Mittwoch U. Sex determination in mythology and history. Arquivos brasileiros de endocrinologia e metabologia 2005; 49: 7-13.
2. Jones OD. Sex selection: regulating technology enabling the predetermination of a child's gender. Harv J Law Technol 1992; 6: 1-62.
3. Levin RJ. Human sex pre-selection. Oxf Rev Reprod Biol 1987; 9: 161-191.
4. Serour GI. Transcultural issues in gender selection. International Congress Series 2004; 1266: 21-31.
5. Shettles LB, Rorvik D. How to Choose the Sex of Your Baby. Ed. Doubleday 1970.
6. Lee J, Paik M. Sex preferences and fertility in South Korea during the year of the Horse. Demography 2006; 43: 269-292.
7. Bandyopadhyay S, Singh AJ. Sex selection through traditional drugs in rural North India. Indian Journal of Community Medicine 2007; 32: 1-3.
8. Wilson EB. Recent Researches on the Determination and Heredity of Sex. Science 1909; 29: 53-70.
9. Wilcox AJ, Weinberg CR, Baird DD. Timing of sexual intercourse in relation to ovulation. Effects on the probability of conception, survival of the pregnancy, and sex of the baby. N Engl J Med 1995; 333: 1517-1521.
10. Herrmann BG, Koschorz B, Wertz K, McLaughlin KJ, Kispert A. A protein kinase encoded by the t complex responder gene causes non-mendelian inheritance. Nature 1999; 402: 141-146.
11. Seidel G. Economics of selecting for sex: the most important genetic trait. Theriogenology 2003; 59: 585-598.
12. Cunningham EP. The effect of changing the sex ratio on the efficiency of cattle breeding operations. Livestock Prod. Sci. 1975; 2: 29-32.
13. Haaland-Holmes K, Gaskins CT, Hillers JK, Clarke SE. Economic benefits of sex ratio control with different mating systems in beef production. Proc. West. Sect. Am. Soc. Anim. Sci. 1982; 33: 228:231.
14. Grant V. Could maternal testosterone levels govern mammalian sex ratio deviations? Journal of Theoretical Biology 2007; 246: 708-719.
15. Trivers RL, Willard DE. Natural selection of parental ability to vary the sex ratio of offspring. Science 1973; 179: 90-92.
16. Rosenfeld CS, Roberts RM. Maternal diet and other factors affecting offspring sex ratio: a review. Biol Reprod 2004; 71: 1063-1070.
17. Wiley DN, Clapham PJ. Does maternal condition affect the sex ratio of offspring in humpback whales? Animal Behaviour 1993; 46: 321-324.
18. Clutton-Brock T, Albon S, Guinness F. Maternal dominance, breeding success and birth sex ratios in red deer. Nature 1984; 308: 358-360.
19. Flint AP, Albon SD, Jafar SI. Blastocyst development and conceptus sex selection in red deer Cervus elaphus: studies of a free-living population on the Isle of Rum. Gen Comp Endocrinol 1997; 106: 374-383.
20. Kojola I, Eloranta E. Influences of maternal body weight, age, and parity on sex ratio in semidomesticated reindeer. Evolution 1989; 43: 1331-1336.
21. Cameron EZ, Linklater WL, Stafford KJ, Veltman CJ. Birth sex ratios related to mare condition at conception in Kaimanawa horses. Behav. Ecol. 1999; 10: 472-475.
22. Roche JR, Lee JM, Berry DP. Pre-conception energy balance and secondary sex ratio--partial support for the Trivers-Willard hypothesis in dairy cows. J Dairy Sci 2006; 89: 2119-2125.
23. Gibson MA, Mace R. Strong mothers bear more sons in rural Ethiopia. Proc Biol Sci 2003; 270 Suppl 1: S108-109.
24. Blumberg MS, Mennella JA, Moltz H, McClintock MK. Facultative sex-ratio adjustment in Norway rats: litters born asynchronously are female biased. Behav. Ecol. Sociobiol. 1992; 31: 401-408.
25. Andersson R, Bergstrom S. Is maternal malnutrition associated with a low sex ratio at birth? Hum Biol 1998; 70: 1101-1106.
26. Cagnacci A, Renzi A, Arangino S, Alessandrini C, Volpe A. Influences of maternal weight on the secondary sex ratio of human offspring. Hum Reprod 2004; 19: 442-444.
27. Blanchard P, Festa-Bianchet M, Gaillard JM, Jorgeson JT. Maternal condition and offspring sex ratio in polygynous ungulates: a case study of bighorn sheep. Behav. Ecol. 2005; 16: 274-279.

28. Cameron EZ, Linklater WL. Extreme sex ratio variation in relation to change in condition around conception. Biol Lett 2007; 3: 395-397.
29. Austad SN, Sunquist ME. Sex-ratio manipulation in the common opossum. Nature 1986; 324: 58-60.
30. Enright WJ, Spicer LJ, Kelly M, Culleton N, Prendiville DJ. Energy level in winter diets of Fallow deer: effect on plasma levels of insulin-like growth factor-I and sex ratio of their offspring. Small Rumin Res 2001; 39: 253-259.
31. Skjervold H, James J. Causes of variation in the sex ratio in dairy cattle. Z. Tierz. Zuechtungsbiol. 1979; 95: 293-305.
32. Williams RJ, Gloster SP. Human sex ratio as it relates to caloric availability. Soc Biol 1992; 39: 285-291.
33. Helle S, Laaksonen T, Adamsson A, Paranko J, Huitu O. Female field voles with high testosterone and glucose levels produce male-biased litters. Animal Behaviour 2007; 2007: doi:10.1016.
34. Machado AF, Zimmerman EF, Hovland DN, Jr., Weiss R, Collins MD. Diabetic embryopathy in C57BL/6J mice. Altered fetal sex ratio and impact of the splotch allele. Diabetes 2001; 50: 1193-1199.
35. Rjasanowski I, Kloting I, Kovacs P. Altered sex ratio in offspring of mothers with insulin-dependent diabetes mellitus. Lancet 1998; 351: 497-498.
36. Meikle DB, Thornton MW. Premating and gestational effects of maternal nutrition on secondary sex ratio in house mice. J Reprod Fertil 1995; 105: 193-196.
37. Rivers JP, Crawford MA. Maternal nutrition and the sex ratio at birth. Nature 1974; 252: 297-298.
38. Meikle DB, Drickamer LC. Food availability and secondary sex ratio variation in wild and laboratory house mice (Mus musculus). J Reprod Fertil 1986; 78: 587-591.
39. Mitra J, Chowdhury M. Glycerylphosphorylcholine diesterase activity of uterine fluid in conditions inducing secondary sex ratio change in the rat. Gamete Res 1989; 23: 415-420.
40. Verme LJ. Reproduction manners of white-tailed deer related to nutritional plane. Journal of Wildlife Management 1969; 33: 881-887.
41. Rosenfeld CS, Grimm KM, Livingston KA, Brokman AM, Lamberson WE, Roberts RM. Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. Proc Natl Acad Sci U S A 2003; 100: 4628-4632.
42. Cameron EZ. Facultative adjustment of mammalian sex ratios in support of the Trivers-Willard hypothesis: evidence for a mechanism. Proc Biol Sci 2004; 271: 1723-1728.
43. Green MP, Spate LD, Parks TE, Kimura K, Murphy CN, Williams JE, Kerley MS, Green JA, Keisler DH, Roberts RM. Nutritional skewing of conceptus sex in sheep: effects of a maternal diet enriched in rumen-protected polyunsaturated fatty acids (PUFA). Reprod Biol Endocrinol 2008; 6: 21.
44. Fountain ED, Mao J, Whyte JJ, Mueller KE, Ellersieck MR, Will MJ, Roberts RM, Macdonald R, Rosenfeld CS. Effects of diets enriched in omega-3 and omega-6 polyunsaturated fatty acids on offspring sex-ratio and maternal behavior in mice. Biol Reprod 2008; 78: 211-217.
45. Bird E, Contreras RJ. Maternal dietary sodium chloride levels affect the sex ratio in rat litters. Physiol Behav 1986; 36: 307-310.
46. Grant V, Metcalf L. Paternal occupation and offspring sex ratio. Sexualities, Evolution and Gender 2003; 5: 191-209.
47. Simpson MJ, Simpson AE. Birth sex ratios and social rank in rhesus monkey mothers. Nature 1982; 300: 440-441.
48. Silk J, Clark-Wheatley C, Rodman P, Samuels A. Differential reproductive success and facultative adjustment of sex ratios among captive female bonnet macaques (*Macaca radiata*). Animal Behaviour 1981; 29: 1106-1120.
49. Meikle D, Drickamer L, Vessey S, Rosenthal T, Fitzgerald K. Maternal dominance rank and secondary sex ratio in domestic swine. Animal Behaviour 1993; 46: 79-85.
50. Kent JP. Birth sex ratios in sheep over nine lambing seasons: years 7-9 and the effects of ageing. Behav. Ecol. Sociobiol. 1995; 36: 101-104.
51. Paul A, Thommen D. Timing of birth, female reproductive success and infant sex ratio in semifree-ranging macaques (*Macaca sylvanus*). Folia Primatologica 1984; 42: 2-16.
52. Van Schaik CP, Netto WJ, Van Amerongen AJJ, Westland H. Social rank and sex ratio of captive long-tailed macaque females (*Macaca fasciculatis*). Am. J. Primatol. 1986; 19: 147-161.

53. Drickamer LC. Social dominance, reproduction, and release of the maturation-delaying chemosignal in the urine of female house mice (Mus musculus). J Comp Psychol 1985; 99: 411-419.
54. Grant VJ. Maternal dominance and the conception of sons. Br J Med Psychol 1994; 67 (Pt 4): 343-351.
55. Packer C, Collins DA, Eberly LE. Problems with primate sex ratios. Philos Trans R Soc Lond B Biol Sci 2000; 355: 1627-1635.
56. Grant VJ, Irwin RJ. Follicular fluid steroid levels and subsequent sex of bovine embryos. J Exp Zool A Comp Exp Biol 2005; 303: 1120-1125.
57. Singh D, Zambarano RJ. Offspring sex ratio in women with android body fat distribution. Hum Biol 1997; 69: 545-556.
58. Brown GR, Silk JB. Reconsidering the null hypothesis: Is maternal rank associated with birth sex ratios in primate groups? Proc Natl Acad Sci U S A 2002; 99: 11252-11255.
59. Rhine RJ, Norton GW, Rogers J, Wasser SK. Secondary sex ratio and maternal dominance rank among wild yellow baboons (*Papio cynocephalus*) of Mikumi National Park, Tanzania. Am. J. Primatol. 1991; 27: 261-273.
60. Rawlins RG, Kessler MJ. Secondary sex ratio variation in the Cayo Santiago macaque population. Am. J. Primatol. 2005; 10: 9-23.
61. Rorie RW, Lester TD, Lindsey BR, McNew RW. Effect of timing of artificial insemination on gender ratio in beef cattle. Theriogenology 1999; 52: 1035-1041.
62. Pratt NC, Huck UW, Lisk RD. Offspring sex ratio in hamsters is correlated with vaginal pH at certain times of mating. In: Behav Neural Biol, vol. 48; 1987: 310-316.
63. Verme LJ, Ozoga JJ. Sex ratio of white-tailed deer and the estrus cycle. J. Wildl. Manage. 1981; 45: 710-715.
64. Gutierrez-Adan A, Perez G, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. Zygote 1999; 7: 37-43.
65. Wehner GR, Wood C, Tague A, Barker D, Hubert H. Efficiency of the OVATEC unit for estrus detection and calf sex control in beef cows. Anim Reprod Sci 1997; 46: 27-34.
66. Martinez F, Kaabi M, Martinez-Pastor F, Alvarez M, Anel E, Boixo JC, de Paz P, Anel L. Effect of the interval between estrus onset and artificial insemination on sex ratio and fertility in cattle: a field study. Theriogenology 2004; 62: 1264-1270.
67. Krackow S, Burgoyne PS. Timing of mating, developmental asynchrony and the sex ratio in mice. Physiol Behav 1997; 63: 81-84.
68. Pursley JR, Silcox RW, Wiltbank MC. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J Dairy Sci 1998; 81: 2139-2144.
69. Harlap S. Gender of infants conceived on different days of the menstrual cycle. N Engl J Med 1979; 300: 1445-1448.
70. Weinberg CR, Baird DD, Wilcox AJ. The sex of the baby may be related to the length of the follicular phase in the conception cycle. Hum Reprod 1995; 10: 304-307.
71. Guerrero R. Sex ratio: a statistical association with the type and time of insemination in the menstrual cycle. Int J Fertil 1970; 15: 221-225.
72. Hedricks C, McClintock MK. Timing of insemination is correlated with the secondary sex ratio of Norway rats. Physiol Behav 1990; 48: 625-632.
73. James WH. The fragile male. Male zygotes are often formed at suboptimal times in fertile cycle. Bmj 2001; 322: 617.
74. Vega MD, Pena AI, Gullon J, Prieto C, Barrio M, Becerra JJ, Herradon PG, Quintela LA. Sex ratio in rabbits following modified artificial insemination. Anim Reprod Sci 2008; 103: 385-391.
75. Soede NM, Nissen AK, Kemp B. Timing of insemination relative to ovulation in pigs: effects on sex ratio of offspring. Theriogenology 2000; 53: 1003-1011.
76. Roelofs JB, Bouwman EB, Pedersen HG, Rasmussen ZR, Soede NM, Thomsen PD, Kemp B. Effect of time of artificial insemination on embryo sex ratio in dairy cattle. Anim Reprod Sci 2006; 93: 366-371.
77. Foote RH. The sex ratio in dairy cattle under various conditions. Theriogenology 1977; 8: 349-356.
78. Vazquez MI, Molina A, Mazón MS, Brito JL, Soto-Camargo R, Martínez RD. Determinación del estado reproductivo del ganado bovino sacrificado en tres rastros municipales del estado de Guerrero. Vet Mex 1993; 24: 155-157.

79. Hylan D, Giraldo AM, Carter JA, Gentry GT, Jr., Bondioli KR, Godke RA. Sex ratio of bovine embryos and calves originating from the left and right ovaries. Biol Reprod 2009; 81: 933-938.
80. Clark MM, Ham M, Galef BG, Jr. Differences in the sex ratios of offspring originating in the right and left ovaries of Mongolian gerbils (Meriones unguiculatus). J Reprod Fertil 1994; 101: 393-396.
81. YoungLai EV, Pan CC, Bhavnani BR. Asymmetric distribution of male and female foetuses in the pregnant rabbit uterus. Experientia 1981; 37: 690-691.
82. Sakai N, Endo A. Effects of induced ovulation by pregnant mare's serum and human chorionic gonadotropin on the sex ratio of mouse fetuses. Gamete Res 1987; 16: 319-322.
83. Clark MM, Galef BG, Jr. Sexual segregation in the left and right horns of the gerbil uterus: "the male embryo is usually on the right, the female on the left" (Hippocrates). Dev Psychobiol 1990; 23: 29-37.
84. Herbert K, Bruce NW. Sequence of implantation and fetal and placental weights in the rat. J Reprod Fertil 1980; 60: 29-32.
85. James WH. The sexes of piglets within the uterine horns. J Hered 1982; 73: 378.
86. Cohen J, Stewart I. That´s amazing, isn´t it? New Scientist 1998; January 17: 24-28.
87. Endo A, Goto T, Sakai N. Distribution by sex of mouse fetuses in the intrauterine position. Gamete Res 1987; 16: 79-82.
88. Lane EA, Hyde TS. Effect of maternal stress on fertility and sex ratio: a pilot study with rats. J Abnorm Psychol 1973; 82: 78-80.
89. Pratt NC, Lisk RD. Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (Mesocricetus auratus). J Reprod Fertil 1989; 87: 763-769.
90. Geiringer E. Effect of ACTH on sex ratio of the albino rat. Proc Soc Exp Biol Med 1961; 106: 752-754.
91. Pratt NC, Lisk RD. Dexamethasone can prevent stress-related litter deficits in the golden hamster. Behav Neural Biol 1990; 54: 1-12.
92. Hansen D, Moller H, Olsen J. Severe periconceptional life events and the sex ratio in offspring: follow up study based on five national registers. Bmj 1999; 319: 548-549.
93. Lyster WR. Altered sex ratio after the London smog of 1952 and the Brisbane flood of 1965. J Obstet Gynaecol Br Commonw 1974; 81: 626-631.
94. Fukuda M, Fukuda K, Shimizu T, Moller H. Decline in sex ratio at birth after Kobe earthquake. Hum Reprod 1998; 13: 2321-2322.
95. Zorn B, Sucur V, Stare J, Meden-Vrtovec H. Decline in sex ratio at birth after 10-day war in Slovenia: brief communication. Hum Reprod 2002; 17: 3173-3177.
96. Catalano R, Bruckner T, Gould J, Eskenazi B, Anderson E. Sex ratios in California following the terrorist attacks of September 11, 2001. Hum Reprod 2005; 20: 1221-1227.
97. Catalano R, Bruckner T, Hartig T, Ong M. Population stress and the Swedish sex ratio. Paediatr Perinat Epidemiol 2005; 19: 413-420.
98. Johnson CN, Clinchy M, Taylor AC, Krebs CJ, Jarman PJ, Payne A, Ritchie EG. Adjustment of offspring sex ratios in relation to the availability of resources for philopatric offspring in the common brushtail possum. Proc Biol Sci 2001; 268: 2001-2005.
99. Clark A. Sex ratio and local resource competition in a prosimian primate. Science 1978; 201: 163-165.
100. MacMahon B, Pugh T. Sex ratio of white births in the United States during the Second World War. American Journal of Human Genetics 1954; 6: 284-292.
101. Graffelman J, Hoekstra R. A statistical analysis of the effect of warfare on the human secondary sex ratio. Human Biology 2000; 72: 433-445.
102. Charnov EL. The theory of sex allocation. Monogr Popul Biol 1982; 18: 1-355.
103. Lummaa V, Merila J, Kause A. Adaptive sex ratio variation in pre-industrial human (Homo sapiens) populations? Proc Biol Sci 1998; 265: 563-568.
104. Mech LD. Disproportionate sex ratios of wolf pups. J. Wildl. Manage. 1975; 39: 737-740.
105. Perret M. Influence of social factors on sex ratio at birth, maternal investment and young survival in a prosimian primate. Behav. Ecol. Sociobiol. 1990; 27: 447-454.
106. Dennis PM, Rajala-Schultz PJ, Funk JA, Blumer ES, Miller RE, Wittum TE, Saville WJ. Risk factors associated with a skewed natal sex ratio in captive black rhinoceroses (Diceros bicornis) in the United States. J Zoo Wildl Med 2007; 38: 533-539.
107. Degayner EJ, Jordan PA. Skewed fetal sex ratios in white-tailed deer: Evidence and Evolutionary speculations. Res. Symp. Natl. Zool. Park. 1987: 178-188.

108. Huck UW, Pratt NC, Labov JB, Lisk RD. Effects of age and parity on litter size and offspring sex ratio in golden hamsters (Mesocricetus auratus). J Reprod Fertil 1988; 83: 209-214.
109. Berry DP, Cromie AR. Artificial insemination increases the probability of a male calf in dairy and beef cattle. Theriogenology 2007; 67: 346-352.
110. Orvos H, Kozinszky Z, Bartfai G. Natural variation in the human sex ratio. Hum Reprod 2001; 16: 803.
111. Nicolich MJ, Huebner WW, Schnatter AR. Influence of parental and biological factors on the male birth fraction in the United States: an analysis of birth certificate data from 1964 through 1988. Fertil Steril 2000; 73: 487-492.
112. Gutierrez-Adan A, Pintado B, de la Fuente J. Demographic and behavioral determinants of the reduction of male-to-female birth ratio in Spain from 1981 to 1997. Hum Biol 2000; 72: 891-898.
113. Garfinkel J, Selvin S. A multivariate analysis of the relationship between parental age and birth order and the human secondary sex ratio. J Biosoc Sci 1976; 8: 113-121.
114. James WH, Rostron J. Parental age, parity and sex ratio in births in England and Wales, 1968-77. J Biosoc Sci 1985; 17: 47-56.
115. Roche JR, Lee JM, Berry DP. Climatic factors and secondary sex ratio in dairy cows. J Dairy Sci 2006; 89: 3221-3227.
116. Peaker M, Taylor E. Sex ratio and litter size in the guinea-pig. J Reprod Fertil 1996; 108: 63-67.
117. Servanty S, Gaillard JM, Allainé D, Brandt S, Baubet E. Litter size and fetal sex ratio adjustment in a hihgly polytocus species: the wild boar. Behav. Ecol. 2006; 18: 427-432.
118. James WH. Was the widespread decline in sex ratios at birth caused by reproductive hazards? Hum Reprod 1998; 13: 1083-1084.
119. Astolfi P, Zonta LA. Reduced male births in major Italian cities. Hum Reprod 1999; 14: 3116-3119.
120. Williams FL, Lawson AB, Lloyd OL. Low sex ratios of births in areas at risk from air pollution from incinerators, as shown by geographical analysis and 3-dimensional mapping. Int J Epidemiol 1992; 21: 311-319.
121. Mocarelli P, Gerthoux PM, Ferrari E, Patterson DG, Jr., Kieszak SM, Brambilla P, Vincoli N, Signorini S, Tramacere P, Carreri V, Sampson EJ, Turner WE, Needham LL. Paternal concentrations of dioxin and sex ratio of offspring. Lancet 2000; 355: 1858-1863.
122. Weisskopf MG, Anderson HA, Hanrahan LP. Decreased sex ratio following maternal exposure to polychlorinated biphenyls from contaminated Great Lakes sport-caught fish: a retrospective cohort study. Environ Health 2003; 2: 2.
123. Beratis NG, Asimacopoulou A, Varvarigou A. Association of secondary sex ratio with smoking and parity. Fertil Steril 2008; 89: 662-667.
124. Fukuda M, Fukuda K, Shimizu T, Andersen CY, Byskov AG. Parental periconceptional smoking and male: female ratio of newborn infants. Lancet 2002; 359: 1407-1408.
125. Mills JL, England L, Granath F, Cnattingius S. Cigarette smoking and the male-female sex ratio. Fertil Steril 2003; 79: 1243-1245.
126. Heron J, Ness A. Lack of association between smoking behavior and the sex ratio of offspring in the avon longitudinal study of parents and children. Fertil Steril 2004; 81: 700-702.
127. Gloria-Bottini F, Meloni GF, Grassi S, Bottini N, Saccucci P, Giarrizzo GF, Magrini A, Bergamaschi A, Bottini E. ACP1 and offspring sex ratio in smoking puerperae: a study at population level. Early Hum Dev 2007; 83: 349-354.
128. Weathersbee PS, Ax RL, Lodge JR. Caffeine-mediated changes of sex ratio in Chinese hamsters, Cricetulus griseus. J Reprod Fertil 1975; 43: 141-143.
129. Mubarak AA, Mubarak AA. Does high voltage electricity have an effect on the sex distribution of offspring? Hum Reprod 1996; 11: 230-231.
130. Davis D, Gottlieb M, Stampnitzky J. Reduced ratio of male to female births in several industrial countries: a sentinel health indicator? JAMA 1998; 279: 1018-1023.
131. Olsson H, Brandt L. Sex ratio in offspring of patients with non-Hodgkin lymphoma. N Engl J Med 1982; 306: 367-368.
132. James WH. Gonadotrophin and the human secondary sex ratio. Br Med J 1980; 281: 711-712.
133. James WH. The sex ratio of offspring of women with congenital adrenal hyperplasia. Hum Reprod 2009; 24: 250-251; author reply 251.
134. Piazze J, Nigro G, Mazzocco M, Marchiani E, Brancato V, Anceschi MM, Cosmi EV. The effect of primary cytomegalovirus infection on fetal lung maturity indices. Early Hum Dev 1999; 54: 137-144.

135. Shields MD, O'Hare B, Nelson J, Stewart MC, Coyle P. Different sex ratios at birth in Europe and North America. Maternal cytomegalovirus seropositivity affects sex determination. Bmj 2002; 325: 334.
136. Chahnazarian A, Blumberg BS, London WT. Hepatitis B and the sex ratio at birth: a comparative analysis of four populations. J Biosoc Sci 1988; 20: 357-370.
137. James WH. Sex ratios of offspring of patients with breast cancer and other endocrine related cancers. Int J Cancer 2006; 119: 2710-2711; author reply 2712.
138. Kankova S, Sulc J, Nouzova K, Fajfrlik K, Frynta D, Flegr J. Women infected with parasite Toxoplasma have more sons. Naturwissenschaften 2007; 94: 122-127.
139. Kankova S, Kodym P, Frynta D, Vavrinova R, Kubena A, Flegr J. Influence of latent toxoplasmosis on the secondary sex ratio in mice. Parasitology 2007; 134: 1709-1717.
140. Helle S, Helama S, Jokela J. Temperature-related birth sex ratio bias in historical Sami: warm years bring more sons. Biol Lett 2008; 4: 60-62.
141. Nonaka K, Desjardins B, Charbonneau H, Legare J, Miura T. Human sex ratio at birth and mother's birth season: multivariate analysis. Hum Biol 1999; 71: 875-884.
142. Cagnacci A, Renzi A, Arangino S, Alessandrini C, Volpe A. Interplay between maternal weight and seasons in determining the secondary sex ratio of human offspring. Fertil Steril 2005; 84: 246-248.
143. Brandriff BF, Gordon LA, Haendel S, Singer S, Moore DH, 2nd, Gledhill BL. Sex chromosome ratios determined by karyotypic analysis in albumin-isolated human sperm. Fertil Steril 1986; 46: 678-685.
144. Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA, Johnson LA. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod 1983; 28: 312-321.
145. Ruder A. Paternal-age and birth-order effect on the human secondary sex ratio. Am J Hum Genet 1985; 37: 362-372.
146. Graffelman J, Fugger EF, Keyvanfar K, Schulman JD. Human live birth and sperm-sex ratios compared. Hum Reprod 1999; 14: 2917-2920.
147. Manning JT, Anderton RH, Shutt M. Parental age gap skews child sex ratio. Nature 1997; 389: 344.
148. Gomendio M, Malo AF, Soler AJ, Fernandez-Santos MR, Esteso MC, Garcia AJ, Roldan ER, Garde J. Male fertility and sex ratio at birth in red deer. Science 2006; 314: 1445-1447.
149. Chandler JE, Steinholt-Chenevert HC, Adkinson RW, Moser EB. Sex ratio variation between ejaculates within sire evaluated by polymerase chain reaction, calving, and farrowing records. J Dairy Sci 1998; 81: 1855-1867.
150. Ward MA, Burgoyne PS. The effects of deletions of the mouse Y chromosome long arm on sperm function--intracytoplasmic sperm injection (ICSI)-based analysis. Biol Reprod 2006; 74: 652-658.
151. Jacobsen R, Bostofte E, Engholm G, Hansen J, Skakkebaek NE, Moller H. Fertility and offspring sex ratio of men who develop testicular cancer: a record linkage study. Hum Reprod 2000; 15: 1958-1961.
152. Moller H. Trends in sex-ratio, testicular cancer and male reproductive hazards: are they connected? Apmis 1998; 106: 232-238; discussion 238-239.
153. Cocco P, Fadda D, Ibba A, Melis M, Tocco MG, Atzeri S, Avataneo G, Meloni M, Monni F, Flore C. Reproductive outcomes in DDT applicators. Environ Res 2005; 98: 120-126.
154. Goldsmith JR, Potashnik G, Israeli R. Reproductive outcomes in families of DBCP-exposed men. Arch Environ Health 1984; 39: 85-89.
155. Garry VF, Holland SE, Erickson LL, Burroughs BL. Male reproductive hormones and thyroid function in pesticide applicators in the Red River Valley of Minnesota. J Toxicol Environ Health A 2003; 66: 965-986.
156. Chang BL, Robbins WA, Wei F, Xun L, Wu G, Li N, Elashoff DA. Boron workers in China: exploring work and lifestyle factors related to boron exposure. Aaohn J 2006; 54: 435-443.
157. Milham S, Jr. Unusual sex ratio of births to carbon setter fathers. Am J Ind Med 1993; 23: 829-831.
158. Viloria T, Rubio MC, Rodrigo L, Calderon G, Mercader A, Mateu E, Meseguer M, Remohi J, Pellicer A. Smoking habits of parents and male: female ratio in spermatozoa and preimplantation embryos. Hum Reprod 2005; 20: 2517-2522.
159. James W. Possible constraints on adaptative variation in sex ratio at birth in humans and other primates. Journal of Theoretical Biology 2006; 238: 383-394.

160. Sas M, Szollosi J. [Sex ratio of children of fathers with spermatic disorders following hormone therapy]. Orv Hetil 1980; 121: 2807-2808.
161. Ishihara K, Warita K, Tanida T, Sugawara T, Kitagawa H, Hoshi N. Does paternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affect the sex ratio of offspring? J Vet Med Sci 2007; 69: 347-352.
162. Hwang SY, Kim WJ, Wee JJ, Choi JS, Kim SK. Panax ginseng improves survival and sperm quality in guinea pigs exposed to 2,3,7,8-tetrachlorodibenzo- p-dioxin. BJU Int 2004; 94: 663-668.
163. Jacobsen R, Bostofte E, Skakkebaek NE, Hansen J, Moller H. Offspring sex ratio of subfertile men and men with abnormal sperm characteristics. Hum Reprod 2000; 15: 2369-2370.
164. Chandler JE, Canal AM, Paul JB, Moser EB. Collection frequency affects percent Y-chromosome bearing sperm, sperm head area and quality of bovine ejaculates. Theriogenology 2002; 57: 1327-1346.
165. Sheldon BC. Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. Am Nat 2004; 163: 40-54.
166. Murahashi K, Bucholtz DC, Nagatani S, Tsukahara S, Tsukamura H, Foster DL, Maeda KI. Suppression of luteinizing hormone pulses by restriction of glucose availability is mediated by sensors in the brain stem. Endocrinology 1996; 137: 1171-1176.
167. Zuelke KA, Brackett BG. Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. Endocrinology 1992; 131: 2690-2696.
168. James WH. Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. J Endocrinol 2008; 198: 3-15.
169. Dominko T, First NL. Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. Theriogenology 1997; 47: 1041-1050.
170. Chapman DL, Wolgemuth DJ. Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. Development 1993; 118: 229-240.
171. Hendriksen PJ. Do X and Y spermatozoa differ in proteins? Theriogenology 1999; 52: 1295-1307.
172. Hendriksen PJ, Hoogerbrugge JW, Themmen AP, Koken MH, Hoeijmakers JH, Oostra BA, van der Lende T, Grootegoed JA. Postmeiotic transcription of X and Y chromosomal genes during spermatogenesis in the mouse. Dev Biol 1995; 170: 730-733.
173. Wang PJ, Page DC, McCarrey JR. Differential expression of sex-linked and autosomal germ-cell-specific genes during spermatogenesis in the mouse. Hum Mol Genet 2005; 14: 2911-2918.
174. Schultz N, Hamra FK, Garbers DL. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci U S A 2003; 100: 12201-12206.
175. Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, Nagai T. Effect of maturation culture period of oocytes on the sex ratio of in vitro fertilized bovine embryos. Journal of Reproduction and Development 2006; 52: 123-127.
176. Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. Biol Reprod 2008; 78: 812-815.
177. Grant V, Chamley L. Sex-sorted sperm and fertility: an alternative view. Biology of Reproduction 2007; 76: 184-188.
178. Goodall H, Roberts AM. Differences in motility of human X- and Y-bearing spermatozoa. J Reprod Fertil 1976; 48: 433-436.
179. Rohde W, Porstmann T, Dorner G. Migration of Y-bearing human spermatozoa in cervical mucus. J Reprod Fertil 1973; 33: 167-169.
180. Lechniak D, Strabel T, Bousquet D, King AW. Sperm pre-incubation prior to insemination affects the sex ratio of bovine embryos produced in vitro. Reprod Domest Anim 2003; 38: 224-227.
181. Penfold LM, Holt C, Holt WV, Welch GR, Cran DG, Johnson LA. Comparative motility of X and Y chromosome-bearing bovine sperm separated on the basis of DNA content by flow sorting. Mol Reprod Dev 1998; 50: 323-327.
182. Kochhar HS, Kochhar KP, Basrur PK, King WA. Influence of the duration of gamete interaction on cleavage, growth rate and sex distribution of in vitro produced bovine embryos. Anim Reprod Sci 2003; 77: 33-49.

183. Iwata H, Shiono H, Kon Y, Matsubara K, Kimura K, Kuwayama T, Monji Y. Effects of modification of in vitro fertilization techniques on the sex ratio of the resultant bovine embryos. Anim Reprod Sci 2008; 105: 234-244.
184. Van Dyk Q, Mahony MC, Hodgen GD. Differential binding of X- and Y-chromosome-bearing human spermatozoa to zona pellucida in vitro. Andrologia 2001; 33: 199-205.
185. Schams D, Schallenberger E, Hoffmann B, Karg H. The oestrous cycle of the cow: hormonal parameters and time relationships concerning oestrus, ovulation, and electrical resistance of the vaginal mucus. Acta Endocrinol (Copenh) 1977; 86: 180-192.
186. Martin JF. Hormonal and behavioral determinants of the secondary sex ratio. Soc Biol 1995; 42: 226-238.
187. Gadella BM, Van Gestel RA. Bicarbonate and its role in mammalian sperm function. Anim Reprod Sci 2004; 82-83: 307-319.
188. Garner DL, Seidel GE, Jr. History of commercializing sexed semen for cattle. Theriogenology 2008; 69: 886-895.
189. Seidel GE, Jr. Overview of sexing sperm. Theriogenology 2007; 68: 443-446.
190. Pinkel D, Garner DL, Gledhill BL, Lake S, Stephenson D, Johnson LA. Flow cytometric determination of the proportions of X- and Y-chromosome-bearing sperm in samples of purportedly separated bull sperm. J Anim Sci 1985; 60: 1303-1307.
191. Hafs HD, Boyd LJ. Sex ratios of calves from inseminations after electrophoresis of sperm. J Anim Sci 1974; 38: 603-604.
192. Luderer AA, Dean WW, Zine AR, Hess DM, Foote RH, Wall RJ. Separation of bovine spermatozoa by density on water insoluble Newtonian gels and their use for insemination. Biol Reprod 1982; 26: 813-824.
193. Iizuka R, Kaneko S, Aoki R, Kobayashi T. Sexing of human sperm by discontinuous Percoll density gradient and its clinical application. Hum Reprod 1987; 2: 573-575.
194. Kaneko S, Yamaguchi J, Kobayashi T, Iizuka R. Separation of human X- and Y-bearing sperm using percoll density gradient centrifugation. Fertil Steril 1983; 40: 661-665.
195. Check JH, Kwirenk D, Katsoff D, Press M, Breen E, Baker A. Male:female sex ratio in births resulting from IVF according to swim-up versus Percoll preparation of inseminated sperm. Arch Androl 1994; 33: 63-65.
196. Kobayashi J, Oguro H, Uchida H, Kohsaka T, Sasada H, Sato E. Assessment of bovine X- and Y-bearing spermatozoa in fractions by discontinuous percoll gradients with rapid fluorescence in situ hybridization. J Reprod Dev 2004; 50: 463-469.
197. Lin SP, Lee RK, Tsai YJ, Hwu YM, Lin MH. Separating X-bearing human spermatozoa through a discontinuous Percoll density gradient proved to be inefficient by double-label fluorescent in situ hybridization. J Assist Reprod Genet 1998; 15: 565-569.
198. Samura O, Miharu N, He H, Okamoto E, Ohama K. Assessment of sex chromosome ratio and aneuploidy rate in motile spermatozoa selected by three different methods. Hum Reprod 1997; 12: 2437-2442.
199. Iwasaki S, Shioya Y, Masuda H, Hanada A, Nakahara T. Sex ratio of early embryos fertilized in vitro with spermatozoa separated by percoll. Theriogenology 1988; 30: 1191-1198.
200. Upreti GC, Riches PC, Johnson LA. Attempted sexing of bovine spermatozoa by fractionation on a Percoll density gradient. Gamete Res 1988; 20: 83-92.
201. Machado GM, Carvalho JO, Filho ES, Caixeta ES, Franco MM, Rumpf R, Dode MA. Effect of Percoll volume, duration and force of centrifugation, on in vitro production and sex ratio of bovine embryos. Theriogenology 2009; 71: 1289-1297.
202. Ericsson RJ, Cassou B, Dapremant G. Isolation of progressively motile mammalian sperm: To select for Y sperm or to improve fertility. Proc. 9th Int. Congr. Anim. Reprod. AI. 1980; III: 286-290.
203. Ericsson RJ, Langevin CN, Nishino M. Isolation of fractions rich in human Y sperm. Nature 1973; 246: 421-424.
204. Beal WE, White LM, Garner DL. Sex ratio after insemination of bovine spermatozoa isolated using a bovine serum albumin gradient. J Anim Sci 1984; 58: 1432-1436.
205. Ross A, Robinson JA, Evans HJ. Failure to confirm separation of X- and Y-bearing human sperm using BSA gradients. Nature 1975; 253: 354-355.
206. Evans JM, Douglas TA, Renton JP. An attempt to separate fractions rich in human Y sperm. Nature 1975; 253: 352-354.
207. Ueda K, Yanagimachi R. Sperm chromosome analysis as a new system to test human X- and Y-sperm separation. Gamete Res 1987; 17: 221-228.

208. Lobel SM, Pomponio RJ, Mutter GL. The sex ratio of normal and manipulated human sperm quantitated by the polymerase chain reaction. Fertil Steril 1993; 59: 387-392.
209. Wang HX, Flaherty SP, Swann NJ, Matthews CD. Discontinuous Percoll gradients enrich X-bearing human spermatozoa: a study using double-label fluorescence in-situ hybridization. Hum Reprod 1994; 9: 1265-1270.
210. Silverman AY, Stephens SR, Drouin MT, Zack RG, Osborne J, Ericsson SA. Female sex selection using clomiphene citrate and albumin separation of human sperm. Hum Reprod 2002; 17: 1254-1256.
211. Beckett TA, Martin RH, Hoar DI. Assessment of the sephadex technique for selection of X-bearing human sperm by analysis of sperm chromosomes, deoxyribonucleic acid and Y-bodies. Fertil Steril 1989; 52: 829-835.
212. Bedford JM, Bibeau AM. Failure of sperm sedimentation to influence the sex ratio of rabbits. J Reprod Fertil 1967; 14: 167-170.
213. Courot M, Esnault C. [Sedimentation of sperm and sex ratio of cattle]. Ann Biol Anim Biochim Biophys 1973; 13: 329-334.
214. Madrid-Bury N, Fernandez R, Jimenez A, Perez-Garnelo S, Moreira PN, Pintado B, de la Fuente J, Gutierrez-Adan A. Effect of ejaculate, bull, and a double swim-up sperm processing method on sperm sex ratio. Zygote 2003; 11: 229-235.
215. Han TL, Flaherty SP, Ford JH, Matthews CD. Detection of X- and Y-bearing human spermatozoa after motile sperm isolation by swim-up. Fertil Steril 1993; 60: 1046-1051.
216. Yan J, Feng HL, Chen ZJ, Hu J, Gao X, Qin Y. Influence of swim-up time on the ratio of X- and Y-bearing spermatozoa. Eur J Obstet Gynecol Reprod Biol 2006; 129: 150-154.
217. De Jonge CJ, Flaherty SP, Barnes AM, Swann NJ, Matthews CD. Failure of multitube sperm swim-up for sex preselection. Fertil Steril 1997; 67: 1109-1114.
218. Muehleis PM, Long SY. The effects of altering the pH of seminal fluid on the sex ratio of rabbit offspring. Fertil Steril 1976; 27: 1438-1445.
219. Blecher SR, Howie R, Li S, Detmar J, Blahut LM. A new approach to immunological sexing of sperm. Theriogenology 1999; 52: 1309-1321.
220. Ali JI, Eldridge FE, Koo GC, Schanbacher BD. Enrichment of bovine X- and Y-chromosome-bearing sperm with monoclonal H-Y antibody-fluorescence-activated cell sorter. Arch Androl 1990; 24: 235-245.
221. Howes EA, Miller NG, Dolby C, Hutchings A, Butcher GW, Jones R. A search for sex-specific antigens on bovine spermatozoa using immunological and biochemical techniques to compare the protein profiles of X and Y chromosome-bearing sperm populations separated by fluorescence-activated cell sorting. J Reprod Fertil 1997; 110: 195-204.
222. Sills ES, Kirman I, Colombero LT, Hariprashad J, Rosenwaks Z, Palermo GD. H-Y antigen expression patterns in human X- and Y-chromosome-bearing spermatozoa. Am J Reprod Immunol 1998; 40: 43-47.
223. Pinkel D, Gledhill BL, Lake S, Stephenson D, Van Dilla MA. Sex preselection in mammals? Separation of sperm bearing Y and "O" chromosomes in the vole Microtus oregoni. Science 1982; 218: 904-906.
224. Morrell JM, Keeler KD, Noakes DE, Mackenzie NM, Dresser DW. Sexing of sperm by flow cytometry. Vet Rec 1988; 122: 322-324.
225. Morrell JM, Dresser DW. Offspring from inseminations with mammalian sperm stained with Hoechst 33342, either with or without flow cytometry. Mutat Res 1989; 224: 177-183.
226. Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 1989; 41: 199-203.
227. Hamano K. Sex preselection in bovine by separation of X- and Y-chromosome bearing spermatozoa. J Reprod Dev 2007; 53: 27-38.
228. Rath D, Moench-Tegeder G, Taylor U, Johnson LA. Improved quality of sex-sorted sperm: A prerequisite for wider commercial application. Theriogenology 2009; 71: 22-29.
229. Hutchison JL, Norman HD. Characterization and usage of sexed semen from US field data. Theriogenology 2009; 71: 48.
230. DeJarnette JM, Nebel RL, Marshall CE. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. Theriogenology 2009; 71: 49-58.
231. Borchersen S, Peacock M. Danish A.I. field data with sexed semen. Theriogenology 2009; 71: 59-63.
232. Seidel GE, Jr. Sperm sexing technology-The transition to commercial application An introduction to the symposium "Update on sexing mammalian sperm". Theriogenology 2009; 71: 1-3.

233. Sieber M, Freeman AE, Kelley DH. Effects of body measurements and weight on calf size and calving difficulty of Holsteins. J Dairy Sci 1989; 72: 2402-2410.
234. Hohenboken WD. Applications of sexed semen in cattle production. Theriogenology 1999; 52: 1421-1433.
235. Slanger WD, Anbil R. A systems analysis approach for studying the effect of reproduction-enhancing biotechnologies on the genetic improvement of beef cattle. J Anim Sci 1987; 65: 901-909.
236. Frijters AC, Mullaart E, Roelofs RM, van Hoorne RP, Moreno JF, Moreno O, Merton JS. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology 2009; 71: 64-67.
237. Bodmer M, Janett F, Hassig M, den Daas N, Reichert P, Thun R. Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions. Theriogenology 2005; 64: 1647-1655.
238. Schenk JL, Seidel GE, Jr. Pregnancy rates in cattle with cryopreserved sexed spermatozoa: effects of laser intensity, staining conditions and catalase. Soc Reprod Fertil Suppl 2007; 64: 165-177.
239. Garner DL. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 2009; 71: 11-21.
240. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR, Gutierrez-Adan A. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod 2008; 78: 761-772.
241. Morton KM, Herrmann D, Sieg B, Struckmann C, Maxwell WM, Rath D, Evans G, Lucas-Hahn A, Niemann H, Wrenzycki C. Altered mRNA expression patterns in bovine blastocysts after fertilisation in vitro using flow-cytometrically sex-sorted sperm. Mol Reprod Dev 2007; 74: 931-940.
242. Palma GA, Olivier NS, Neumuller C, Sinowatz F. Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts. Anat Histol Embryol 2008; 37: 67-73.
243. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 2004; 101: 5880-5885.
244. Spinaci M, Merlo B, Zannoni A, Iacono E, De Ambrogi M, Turba ME, Zambelli D. In vitro production of cat blastocysts of predetermined sex using flow cytometrically sorted semen. Theriogenology 2007; 67: 872-877.
245. Meyers MA, Burns G, Arn D, Schenk JL. Birth of canine offspring following insemination of a bitch with flow-sorted spermatozoa. Reprod Fertil Dev 2008; 20: 213.
246. Abeydeera LR, Johnson LA, Welch GR, Wang WH, Boquest AC, Cantley TC, Rieke A, Day BN. Birth of piglets preselected for gender following in vitro fertilization of in vitro matured pig oocytes by X and Y chromosome bearing spermatozoa sorted by high speed flow cytometry. Theriogenology 1998; 50: 981-988.
247. Buchanan BR, Seidel GE, Jr., McCue PM, Schenk JL, Herickhoff LA, Squires EL. Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenology 2000; 53: 1333-1344.
248. Catt SL, Catt JW, Gomez MC, Maxwell WM, Evans G. Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. Vet Rec 1996; 139: 494-495.
249. O'Brien JK, Hollinshead FK, Evans KM, Evans G, Maxwell WM. Flow cytometric sorting of frozen-thawed spermatozoa in sheep and non-human primates. Reprod Fertil Dev 2003; 15: 367-375.
250. Fugger EF. Clinical experience with flow cytometric separation of human X- and Y-chromosome bearing sperm. Theriogenology 1999; 52: 1435-1440.
251. Karabinus DS. Flow cytometric sorting of human sperm: MicroSort((R)) clinical trial update. Theriogenology 2009; 71: 74-79.
252. O'Brien JK, Steinman KJ, Robeck TR. Application of sperm sorting and associated reproductive technology for wildlife management and conservation. Theriogenology 2009; 71: 98-107.
253. McClure PA. Sex-Biased Litter Reduction in Food-Restricted Wood Rats (Neotoma floridana). Science 1981; 211: 1058-1060.

254. Koskela E, Mappes T, Niskanen T, Rutkowska J. Maternal investment in relation to sex ratio and offspring number in a small mammal - a case for Trivers and Willard theory? J Anim Ecol 2009; 78: 1007-1014.
255. Xu KP, Yadav BR, King WA, Betteridge KJ. Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 1992; 31: 249-252.
256. Vatten LJ, Skjaerven R. Offspring sex and pregnancy outcome by length of gestation. Early Hum Dev 2004; 76: 47-54.
257. Garcia-Rodriguez J, Garcia-Martin M, Nogueras-Ocana M, de Dios Luna-del-Castillo J, Espigares Garcia M, Olea N, Lardelli-Claret P. Exposure to pesticides and cryptorchidism: geographical evidence of a possible association. Environ Health Perspect 1996; 104: 1090-1095.
258. Garry VF, Schreinemachers D, Harkins ME, Griffith J. Pesticide appliers, biocides, and birth defects in rural Minnesota. Environ Health Perspect 1996; 104: 394-399.
259. Varvarigou AA, Asimakopoulou A, Beratis NG. Impact of maternal smoking on birth size: effect of parity and sex dimorphism. Neonatology 2009; 95: 61-67.
260. James WH. Periconceptual parental smoking and sex ratio of offspring. Lancet 2002; 360: 1515; author reply 1515-1516.
261. Catalano R, Bruckner T, Anderson E, Gould JB. Fetal death sex ratios: a test of the economic stress hypothesis. Int J Epidemiol 2005; 34: 944-948.
262. Bacon SJ, McClintock MK. Sex ratio bias in postpartum-conceived Norway rat litters is produced by embryonic loss in midpregnancy. J Reprod Fertil 1999; 117: 403-411.
263. Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J Reprod Fertil 1991; 93: 125-132.
264. Ray PF, Conaghan J, Winston RM, Handyside AH. Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. J Reprod Fertil 1995; 104: 165-171.
265. Kimura K, Spate LD, Green MP, Roberts RM. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Mol Reprod Dev 2005; 72: 201-207.
266. Gutierrez-Adan A, Perez-Crespo M, Fernandez-Gonzalez R, Ramirez MA, Moreira P, Pintado B, Lonergan P, Rizos D. Developmental consequences of sexual dimorphism during pre-implantation embryonic development. Reprod Domest Anim 2006; 41 Suppl 2: 54-62.
267. Bredbacka K, Bredbacka P. Glucose controls sex-related growth rate differences of bovine embryos produced in vitro. J Reprod Fertil 1996; 106: 169-172.
268. Gutierrez-Adan A, Oter M, Martinez-Madrid B, Pintado B, De La Fuente J. Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. Mol Reprod Dev 2000; 55: 146-151.
269. Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol Reprod 2002; 66: 127-134.
270. Jimenez A, Madrid-Bury N, Fernandez R, Perez-Garnelo S, Moreira P, Pintado B, De la Fuente J, Gutierrez-Adan A. Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. Molecular Reproduction and Development 2003; 65: 180-187.
271. Taylor DM, Handyside AH, Ray PF, Dibb NJ, Winston RM, Ao A. Quantitative measurement of transcript levels throughout human preimplantation development: analysis of hypoxanthine phosphoribosyl transferase. Mol Hum Reprod 2001; 7: 147-154.
272. Perez-Crespo M, Ramirez MA, Fernandez-Gonzalez R, Rizos D, Lonergan P, Pintado B, Gutierrez-Adan A. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol Reprod Dev 2005; 72: 502-510.
273. Cameron EZ, Lemons PR, Bateman PW, Bennett NC. Experimental alteration of litter sex ratios in a mammal. Proc Biol Sci 2008; 275: 323-327.
274. Kimura K, Iwata H, Thompson JG. The effect of glucosamine concentration on the development and sex ratio of bovine embryos. Anim Reprod Sci 2008; 103: 228-238.
275. Jost A. Problems of fetal endocrinology: the gonadal and hypophyseal hormones. Rec Progr Horm Res 1953; 8: 379-418.
276. Mittwoch U. Blastocysts prepare for the race to be male. Hum Reprod 1993; 8: 1550-1555.

277. Nedambale TL, Dinnyes A, Yang X, Tian XC. Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. Biol Reprod 2004; 71: 1671-1676.
278. Valdivia RP, Kunieda T, Azuma S, Toyoda Y. PCR sexing and developmental rate differences in preimplantation mouse embryos fertilized and cultured in vitro. Mol Reprod Dev 1993; 35: 121-126.
279. Cassar G, King WA, King GJ. Influence of sex on early growth of pig conceptuses. J Reprod Fertil 1994; 101: 317-320.
280. Bernardi ML, Delouis C. Sex-related differences in the developmental rate of in-vitro matured/in-vitro fertilized ovine embryos. Hum Reprod 1996; 11: 621-626.
281. Avery B, Madison V, Greve T. Sex and development in bovine in-vitro fertilized embryos. Theriogenology 1991; 35: 953-963.
282. Yadav BR, King WA, Betteridge KJ. Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated in vitro. Mol Reprod Dev 1993; 36: 434-439.
283. Carvalho RV, Del Campo MR, Palasz AT, Plante Y, Mapletoft RJ. Survival rates and sex ratio of bovine IVE embryos frozen at different developmental stages on day 7. Theriogenology 1996; 45: 489-498.
284. Pegoraro LM, Thuard JM, Delalleau N, Guerin B, Deschamps JC, Marquant Le Guienne B, Humblot P. Comparison of sex ratio and cell number of IVM-IVF bovine blastocysts co-cultured with bovine oviduct epithelial cells or with Vero cells. Theriogenology 1998; 49: 1579-1590.
285. Pergament E, Fiddler M, Cho N, Johnson D, Holmgren WJ. Sexual differentiation and preimplantation cell growth. Hum Reprod 1994; 9: 1730-1732.
286. Ng E, Claman P, Leveille MC, Tanphaichitr N, Compitak K, Suwajanakorn S, Wells G. Sex ratio of babies is unchanged after transfer of fast- versus slow-cleaving embryos. J Assist Reprod Genet 1995; 12: 566-568.
287. Fanchin R, Righini C, Olivennes F, Lejeune V, Volante M, Frydman R. Female and male human embryo growth rates are similar before the eight-cell stage. Am J Obstet Gynecol 1998; 178: 45-49.
288. Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology 1998; 50: 1285-1299.
289. Pomp D, Good BA, Geisert RD, Corbin CJ, Conley AJ. Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or -11 pig embryos. J Anim Sci 1995; 73: 1408-1415.
290. Kaminski MA, Ford SP, Youngs CR, Conley AJ. Lack of effect of sex on pig embryonic development in vivo. J Reprod Fertil 1996; 106: 107-110.
291. Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J Reprod Fertil 1999; 117: 159-167.
292. Peippo J, Bredbacka P. Sex-related growth rate differences in mouse preimplantation embryos in vivo and in vitro. Mol Reprod Dev 1995; 40: 56-61.
293. Gutierrez-Adan A, Behboodi E, Andersen GB, Medrano JF, Murray JD. Relationship between stage of development and sex of bovine IVM-IVF embryos cultured in vitro versus in the sheep oviduct. Theriogenology 1996; 46: 515-525.
294. Grisart B, Massip A, Collette L, Dessy F. The sex ratio of bovine embryos produced in vitro in serum-free oviduct cell-conditioned medium is not altered. Theriogenology 1995; 43: 1097-1106.
295. Lansac J, Thepot F, Mayaux MJ, Czyglick F, Wack T, Selva J, Jalbert P. Pregnancy outcome after artificial insemination or IVF with frozen semen donor: a collaborative study of the French CECOS Federation on 21,597 pregnancies. Eur J Obstet Gynecol Reprod Biol 1997; 74: 223-228.
296. Tarin JJ, Bernabeu R, Baviera A, Bonada M, Cano A. Sex selection may be inadvertently performed in in-vitro fertilization-embryo transfer programmes. Hum Reprod 1995; 10: 2992-2998.
297. Quintans CJ, Donaldson MJ, Blanco LA, Sergio Pasqualini R. Deviation in sex ratio after selective transfer of the most developed cocultured blastocysts. J Assist Reprod Genet 1998; 15: 403-404.
298. Menezo YJ, Chouteau J, Torello J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. Fertil Steril 1999; 72: 221-224.

299. Dumoulin JC, Derhaag JG, Bras M, Van Montfoort AP, Kester AD, Evers JL, Geraedts JP, Coonen E. Growth rate of human preimplantation embryos is sex dependent after ICSI but not after IVF. Hum Reprod 2005; 20: 484-491.
300. Pedersen JF. Ultrasound evidence of sexual difference in fetal size in first trimester. Br Med J 1980; 281: 1253.
301. Hoelker M, Schmoll F, Schneider H, Rings F, Gilles M, Tesfaye D, Jennen D, Tholen E, Griese J, Schellander K. Bovine blastocyst diameter as a morphological tool to predict embryo cell counts, embryo sex, hatching ability and developmental characteristics after transfer to recipients. Reprod Fertil Dev 2006; 18: 551-557.
302. Krco CJ, Goldberg EH. H-Y male antigen: detection on eight-cell mouse embryos. Science 1976; 193: 1134-1135.
303. Epstein CJ, Smith S, Travis B. Expression of H-Y antigen on preimplantation mouse embryos. Tissue Antigens 1980; 15: 63-67.
304. Cao QP, Gaudette MF, Robinson DH, Crain WR. Expression of the mouse testis-determining gene Sry in male preimplantation embryos. Mol Reprod Dev 1995; 40: 196-204.
305. Boyer TR, Erickson RP. Detection of circular and linear transcripts of Sry in pre-implantation mouse embryos: differences in requirement for reverse transcriptase. Biochem Biophys Res Commun 1994; 198: 492-496.
306. Gutierrez-Adan A, Behboodi E, Murray JD, Anderson GB. Early transcription of the SRY gene by bovine preimplantation embryos. Mol Reprod Dev 1997; 48: 246-250.
307. Fiddler M, Abdel-Rahman B, Rappolee DA, Pergament E. Expression of SRY transcripts in preimplantation human embryos. Am J Med Genet 1995; 55: 80-84.
308. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N. Reactivation of the paternal X chromosome in early mouse embryos. Science 2004; 303: 666-669.
309. Burgoyne PS, Thornhill AR, Boudrean SK, Darling SM, Bishop CE, Evans EP. The genetic basis of XX-XY differences present before gonadal sex differentiation in the mouse. Philos Trans R Soc Lond B Biol Sci 1995; 350: 253-260 discussion 260-251.
310. Hartshorn C, Rice JE, Wangh LJ. Developmentally-regulated changes of Xist RNA levels in single preimplantation mouse embryos, as revealed by quantitative real-time PCR. Mol Reprod Dev 2002; 61: 425-436.
311. Latham KE, Rambhatla L. Expression of X-linked genes in androgenetic, gynogenetic, and normal mouse preimplantation embryos. Dev Genet 1995; 17: 212-222.
312. Kay GF, Barton SC, Surani MA, Rastan S. Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development. Cell 1994; 77: 639-650.
313. Peippo J, Farazmand A, Kurkilahti M, Markkula M, Basrur PK, King WA. Sex-chromosome linked gene expression in in-vitro produced bovine embryos. Mol Hum Reprod 2002; 8: 923-929.
314. Larson MA, Kimura K, Kubisch HM, Roberts RM. Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-tau. Proc Natl Acad Sci U S A 2001; 98: 9677-9682.
315. Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. Am J Reprod Immunol 1997; 37: 412-420.
316. Kimura K, Spate LD, Green MP, Murphy CN, Seidel GE, Jr., Roberts RM. Sexual dimorphism in interferon-tau production by in vivo-derived bovine embryos. Mol Reprod Dev 2004; 67: 193-199.
317. Kimura K, Spate LD, Green MP, Roberts RM. Effects of oxidative stress and inhibitors of the pentose phosphate pathway on sexually dimorphic production of IFN-tau by bovine blastocysts. Mol Reprod Dev 2004; 68: 88-95.
318. Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, Nakanishi T, Ikawa M, Hamada H, Abe K, Okabe M. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. Curr Biol 2006; 16: 166-172.
319. Bird A. Perceptions of epigenetics. Nature 2007; 447: 396-398.
320. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33 Suppl: 245-254.
321. Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo. Mol Reprod Dev 1996; 44: 476-485.

322. Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 1996; 106: 299-306.
323. Bermejo-Alvarez P, Lonergan P, Rizos D, Gutiérrez-Adán A. Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolisis. Reproductive Biomedicine Online 2010; in press.
324. Bavister BD, Squirrell JM. Mitochondrial distribution and function in oocytes and early embryos. Hum Reprod 2000; 15 Suppl 2: 189-198.
325. May-Panloup P, Vignon X, Chretien MF, Heyman Y, Tamassia M, Malthiery Y, Reynier P. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. Reprod Biol Endocrinol 2005; 3: 65.
326. Mittwoch U. The elusive action of sex-determining genes: mitochondria to the rescue? J Theor Biol 2004; 228: 359-365.
327. Blackburn EH. Structure and function of telomeres. Nature 1991; 350: 569-573.
328. Liu L, Bailey S, Okuka M, Muñoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, Blasco M, Keefe D. Telemore lengthening early in development. Nature Cell Biology 2007; 9: 1436-1441.
329. Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL. Telomere length is reset during early mammalian embryogenesis. Proc Natl Acad Sci U S A 2004; 101: 8034-8038.

OBJETIVES/OBJETIVOS

OBJECTIVES

General objective

To analyze possible sex ratio control preconceptional mechanisms and to determine sex-related physiological, transcriptional and epigenetic differences in bovine preimplantation embryos.

Specific objectives

1) To examine the effect of oocyte maturation for 16 v. 24 h on the kinetics of first cleavage and embryo yield, and on the sex ratio of bovine embryos produced by IVF.
2) To determine whether *in vitro* matured oocytes preferentially select X- or Y-bearing spermatozoa following *in vitro* fertilization, by examining the differences in fertility between sorted (i.e. X- or Y-sorted) versus sorted/recombined (i.e. a mix of X- and Y-sorted sperm) sperm.
3) To examine the differences in kinetics of first cleavage and blastocysts development and relative transcript abundance of developmental-related genes between embryos derived from sorted v. unsorted spermatozoa.
4) To examine differences in kinetics of development and survival under *in vitro* conditions for bovine embryos produced *in vitro*.
5) To analyze sex-related differences in mtDNA content, telomere length, methylation of different regions of the genome, and transcription of genes related with cytosine methylation and histone methylation in bovine blastocysts produced *in vitro*.
6) To study preimplantational sexual dimorphism at the transcriptional level by microarray gene expression profiling of bovine blastocysts produced *in vitro*, and to determine if the sexual dimorphism also occurs for *in vivo* embryos.
7) To determine possible imprinting mechanisms affecting the X-chromosome.

OBJETIVOS

Objetivo general

Analizar los posibles mecanismos preconcepcionales de control de la proporción de sexos y determinar las diferencias fisiológicas, transcripcionales y epigenéticas entre embriones preimplantacionales bovinos de distinto sexo.

Objetivos específicos

1) Examinar el efecto de la maduración del ovocito durante 16 o 24 horas en la cinética de la primera división y en la producción de embriones, y sobre la proporción de sexos de los embriones bovinos producidos por FIV.
2) Determinar si los ovocitos madurados *in vitro* pueden seleccionar a los espermatozoides portadores del cromosoma X o Y durante la fecundación *in vitro*, mediante el examen de las diferencias en la capacidad fecundante entre semen sexado (X o Y) y una mezcla de semen sexado (X e Y).
3) Examinar las diferencias en cinética de la primera división y desarrollo a blastocisto, y en la abundancia relativa de transcritos de genes relacionados con el desarrollo entre embriones obtenidos mediante espermatozoides sexados o sin sexar.
4) Examinar las diferencias en cinética de desarrollo y supervivencia en las condiciones de cultivo *in vitro* entre embriones bovinos de distinto sexo producidos *in vitro*.
5) Analizar las diferencias en contenido de ADN mitocondrial, longitud telomérica, metilación de diferentes regiones genómicas y transcripción de genes relacionados con metilación de citosinas e histonas entre blastocistos bovinos producidos *in vitro*.
6) Estudiar el dimorfismo sexual preimplantacional a nivel transcripcional mediante perfiles de expresión génica de *microarray* en blastocistos bovinos producidos *in vitro*, y determinar si el dimorfismo sexual también ocurre *in vivo*.
7) Determinar posibles mecanismos de impronta genómica parental que afecten al cromosoma X.

CHAPTER 1

EFFECT OF DURATION OF OOCYTE MATURATION ON THE KINETICS OF CLEAVAGE, EMBRYO YIELD AND SEX RATIO IN CATTLE

CSIRO PUBLISHING

Reproduction, Fertility and Development, 2008, **20**, 734–740 www.publish.csiro.au/journals/rfd

Effect of duration of oocyte maturation on the kinetics of cleavage, embryo yield and sex ratio in cattle

Dimitrios Rizos[A,C], *Pablo Bermejo-Alvarez*[A], *Alfonso Gutierrez-Adan*[A] *and Patrick Lonergan*[B]

[A]Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain.

[B]School of Agriculture, Food Science and Veterinary Medicine, College of Life Science, University College, Dublin, Ireland.

[C]Corresponding author. Email: drizos@inia.es

Abstract. The aim of the present study was to examine the effect of maturation for 16 *v.* 24 h on the kinetics of development and the sex ratio of bovine embryos. Oocytes were inseminated at 16 or 24 h after the beginning of maturation using frozen–thawed bull semen. Two-cell embryos at 24, 28, 32, 36, 40, 44 and 48 h post-insemination (hpi) and blastocysts at Days 6, 7 and 8 from both groups were snap-frozen individually and stored at −80°C until determination of embryo sex. Insemination at 16 h resulted in a lower cleavage rate at 48 hpi than insemination at 24 h (70.6% *v*. 77.1%, respectively, $P < 0.05$). In terms of the evolution of cleavage divisions, insemination at 24 h resulted in a typical pattern of cleavage such that by 32 hpi, ~58% of presumptive zygotes had cleaved. In contrast, first cleavage following insemination at 16 h was significantly slower such that by 32 hpi, ~35% of presumptive zygotes had cleaved. Duration of IVM did not affect blastocyst yield (~37%). The overall sex ratio of 2-cell embryos at 48 hpi differed from 1 : 1 in favour of males in both groups (24 h: 55.9 *v*. 44.1%; 16 h: 59.1 *v*. 40.9%, $P < 0.05$). Similarly, the overall sex ratio of blastocysts differed from 1 : 1 in both groups (24 h: 59.7 *v*. 40.3%; 16 h: 58.5 *v*. 41.5%, $P < 0.05$). In conclusion, timing of gamete interaction and maturity of the oocyte at the time of the interaction can affect the kinetics of the early cleavage divisions but has no effect on the sex ratio of the embryos produce.

Additional keywords: IVM, PCR.

Introduction

The ability to preferentially produce either male or female offspring would be of enormous economic benefit to the livestock industry. The only consistent approach to achieving this is the use of sex-sorted sperm, however, problems with low throughput of sperm have thus far limited its widespread use (Seidel 2003, 2007; Garner and Seidel 2008). Using unsorted sperm, several factors have been shown to influence sex ratio in cattle including (*1*) timing of insemination (Wehner *et al.* 1997; Pursley *et al.* 1998; Martinez *et al.* 2004), (*2*) the maturational state of the oocyte at the time of insemination *in vitro* (Dominko and First 1997; Agung *et al.* 2006), (*3*) the duration of gamete co-incubation *in vitro* (Kochhar *et al.* 2003) and (*4*) the post-fertilisation culture conditions *in vitro* (Gutiérrez-Adán *et al.* 1996, 2001*a*, 2001*b*).

Studies in a variety of mammals suggest that the timing of mating or insemination in relation to ovulation can influence the offspring sex ratio, with early insemination resulting in more females and late insemination more males (reviewed by Rorie 1999). Wehner *et al*. (1997) reported that insemination early during oestrus (20 h before ovulation) resulted in 93% female calves whilst late insemination (10 h before ovulation) resulted in 92% males. Similarly, Martinez *et al*. (2004) reported >70% female calves following early insemination (8–18 h after oestrus onset) compared with >70% male calves if insemination took place late (after 30 h). In sheep, Gutiérrez-Adán *et al*. (1999) reported that insemination of ewes during the 5 h preceding ovulation led to more female offspring compared with insemination during the 5 h after ovulation, which resulted in more males. While the existence of this phenomenon is not universally accepted, proposed mechanisms include preferential transport of X- or Y-bearing sperm in the female tract, preferential selection of sperm at the site of fertilisation or sex-specific embryo mortality after fertilisation. However, others have failed to replicate these results (Jobst and Nebel 1998; Rorie *et al.* 1999; Roelofs *et al.* 2006).

The maturational state of the oocyte at the time of insemination *in vitro* has been shown to influence the sex ratio of the resulting embryos. Dominko and First (1997) and Gutiérrez-Adán *et al*. (1999) selected bovine oocytes at 16 h for the presence of a polar body and either inseminated them immediately or after an 8-h delay at 24 h. Delayed insemination enhanced the cleavage rate and proportion developing to the 8-cell stage as well as significantly increasing the proportion of male embryos. It is not known whether such a deviation in sex ratio would exist

 10.1071/RD08083 1031-3613/08/060734

in unselected oocytes. Therefore, the aim of the present study was to examine the effect of maturation for 16 *v.* 24 h on the kinetics of development after insemination and the sex ratio of bovine embryos. *In vitro* embryo production in cattle is a rather inefficient process; despite high rates of maturation and fertilisation (cleavage) on average only ~30–40% of immature oocytes recovered from abattoir-derived ovaries develop to the blastocyst stage. Because of this substantial loss of embryos between fertilisation and blastocyst formation a more valid estimation of the sex ratio could be obtained by sexing the embryos shortly after fertilisation. Thus, we assessed the effect of maturation length on sex ratio at both the 2-cell and blastocyst stage. Indirectly, this also allowed an assessment of whether postfertilisation culture conditions preferentially supported more males than females as has been suggested for some culture conditions (Gutiérrez-Adán *et al*. 2001*a*, 2001*b*).

Materials and methods

In vitro *embryo production*

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (Poole, UK). The techniques for producing embryos *in vitro* have been described previously (Rizos *et al.* 2002*a*). Immature cumulus oocyte complexes ($n = 4402$) in 16 replicates (replicate = days of ovary collection) were obtained by aspirating follicles from the ovaries of heifers and cows collected at slaughter and were split into two groups for maturation. Maturation took place in TCM-199 (catalogue no. M4530) supplemented with 10% (v/v) fetal calf serum (FCS, catalogue no. F2442) and 10 ng mL^{-1} epidermal growth factor (catalogue no. E4127) at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. One group ($n = 2198$) was inseminated 16 h after the beginning of maturation and the other at 24 h ($n = 2204$) using frozen–thawed percoll-separated bull sperm (GE Healthcare Bio-sciences, Uppsala, Sweden) at a concentration of 10^6 spermatozoa mL^{-1}. Gametes were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At ~20 h post insemination (hpi), presumptive zygotes were denuded and transferred to 25-μL culture droplets (1 embryo per μL) under mineral oil. Culture took place in SOF + 5% FCS. Plates were incubated at 39°C under an atmosphere of 5% CO_2, 5% O_2 in air with maximum humidity. At 24, 28, 32, 36, 40, 44 and 48 h post insemination all culture dishes were examined for the presence of 2-cell embryos. In half of the replicates ($n = 8$) all 2-cell embryos at each time point were removed from culture, snap-frozen individually in liquid nitrogen and stored at −80°C until determination of embryo sex. Embryos in the remaining 8 replicates were further cultured and all blastocysts were removed at Days 6, 7 and 8 and similarly processed for sex determination.

Embryo sexing by PCR

In order to remove any potential confounding effects of accessory spermatozoa attached to the zona pellucida, all embryos for sexing were removed from culture drops, washed in PBS and then transferred into a 5 mg mL^{-1} pronase solution (Sigma P5147, Madrid, Spain) for 1 min. They were then washed 3 to 4 times in PBS and snap-frozen individually in liquid nitrogen in 0.2-μL eppendorf tubes and stored at −80°C until analysis. For increasing PCR efficiency, embryos were digested with 8 μL per individual 2- to 4-cell embryo or 16 μL per individual blastocyst of a 100 μg mL^{-1} proteinase K (Sigma, P8044–1G) solution at 55°C overnight. After digestion, proteinase K was inactivated at 95°C for 10 min. Two sets of PCR primers were used to determine embryo sex: Y-chromosome specific primers (BRY1a), and bovine-specific satellite sequence primers (Sat1) (Manna *et al.* 2003). The PCR were conducted in a total volume of 25 μL containing 8 μL of the proteinase K-digested sample, 1× Gotaq Flexi buffer, 1 IU of Gotaq (Promega, Madison, WI, USA), 1.25 mM $MgCl_2$, 0.1 mM dNTP, 1 ng μL^{-1} BRY primers and 0.2 ng μL^{-1} Sat primers. PCR was performed with a first cycle (94°C for 3 min, 60°C for 40 s and 72°C for 15 s) followed by 35 cycles (94°C for 15 s, 60°C for 30 s, 72°C for 15 s; final elongation 72°C for 5 min) (Bermejo-Alvarez *et al.* 2008). Products were visualised on an ethidium bromide-stained 2% agarose gel. The gel was visualised under ultraviolet illumination for the positive 300-bp band of BRY 1a and the 216-bp band of the satellite sequence. Samples which exhibited both bands were assigned as male while samples exhibiting only a satellite sequence band were assigned as female. Every PCR was carried out with three controls: male genomic DNA, female genomic DNA and a negative control.

Statistical analysis

Data were analysed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student-Newman-Kleus method) was used for the analysis of cleavage rate and blastocyst yield. The sex ratio of 2-cell embryos and blastocysts was compared with the expected ratio of 1 : 1 using the chi-square test of goodness-of-fit. The effect of time of first cleavage and sex ratio was also analysed using logistic regression (sex regressed on IVM duration and time).

Results

Kinetics of cleavage and embryo development

Insemination at 16 h after initiation of IVM resulted in a lower cleavage rate at 48 hpi than insemination at 24 h (70.6% *v.* 77.1%, respectively, $P < 0.05$). In terms of the evolution of cleavage divisions, insemination at the normal time (24 h after start of IVM) resulted in a typical pattern of cleavage; very few zygotes had cleaved by 24 hpi (2%), a further 27% had cleaved by 28 hpi with another 29% cleaved by 32 hpi, such that by 32 hpi ~58% of presumptive zygotes had cleaved. Put another way, 74% of all zygotes that cleaved had done so by 32 hpi (Table 1; Fig. 1).

In contrast, the speed of oocyte cleavage after IVF was significantly slower when oocytes were inseminated 16 h after the start of IVM; 1% had cleaved by 24 hpi, a further 9% had cleaved by 28 hpi with another 25% cleaved by 32 hpi, such that by 32 hpi ~35% of presumptive zygotes had cleaved, representing 49% of all zygotes which cleaved (Table 1; Fig. 1).

Irrespective of duration of maturation, most blastocysts appeared on Day 6 and Day 7 after insemination (~40–45% of the total blastocyst yield on each of the two days) with a smaller number of additional blastocysts (~11% of total) on

Table 1. Effect of duration of *in vitro* maturation of bovine oocytes on kinetics of cleavage after fertilisation *in vitro*
Sixteen replicates. Data are reported as *n* (% (mean ± s.e.m.)) for each time point and IVM duration

Duration of IVM	No. of COC	24 h	28 h	32 h	36 h	40 h	44 h	48 h
Cumulative[A]								
16 h	2198	23 (1.1 ± 0.2)	218[a] (10.0 ± 1.5)	770[a] (35.3 ± 1.5)	1207[a] (54.8 ± 1.8)	1394[a] (63.3 ± 1.8)	1505 (68.4 ± 1.9)	1557[a] (70.6 ± 1.8)
24 h	2204	47 (2.3 ± 0.9)	630[b] (28.8 ± 3.6)	1267[b] (57.6 ± 3.3)	1486[b] (67.4 ± 2.8)	1586[b] (72.0 ± 2.5)	1650 (74.7 ± 2.4)	1702[b] (77.1 ± 2.2)
At each time point[B]								
16 h	2198	23 (1.1 ± 0.9)	195[a] (8.9 ± 1.5)	552[a] (25.3 ± 1.6)	437[a] (19.6 ± 1.7)	177[a] (8.2 ± 0.8)	110[a] (5.0 ± 0.4)	52 (2.3 ± 0.3)
24 h	2204	47 (2.3 ± 0.9)	583[b] (26.6 ± 3.2)	637[b] (28.9 ± 2.2)	219[b] (9.8 ± 1.3)	100[b] (4.6 ± 0.5)	64[b] (2.7 ± 0.5)	52 (2.5 ± 0.6)

[A]Data represent the cumulative total number of oocytes cleaved at each observation time.
[B]Data represent number of oocytes cleaving since previous observation time.
[a,b]Indicates a significant difference ($P < 0.05$) between treatments at a particular time point.

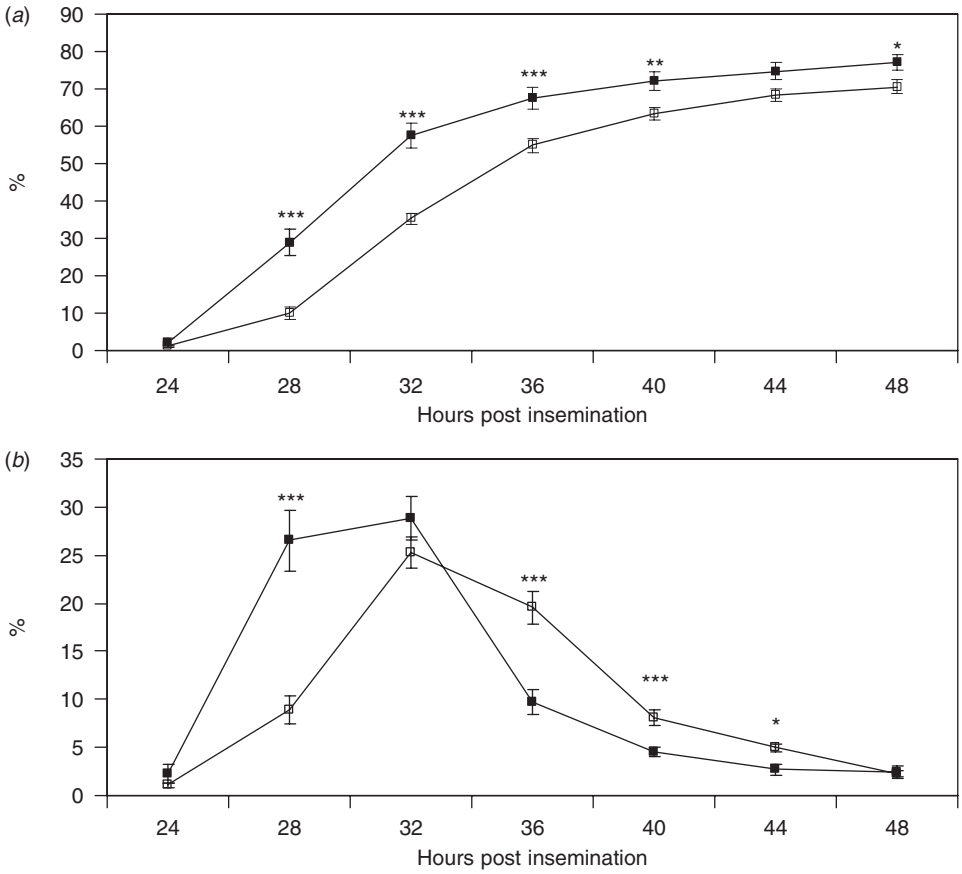


Fig. 1. Kinetics of first cleavage division in bovine zygotes after IVF following oocyte maturation for 16 (□, $n = 2198$) or 24 h (■, $n = 2204$). (*a*) Cumulative cleavage. (*b*) Proportion of zygotes cleaving at each time point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (16 replicates).

Day 8. Duration of IVM did not affect the yield of blastocysts (Table 2; Fig. 2).

Sex ratio of bovine 2-cell embryos and blastocysts

Treatment did not affect the sex ratio of 2-cell embryos at any of the time points studied, apart from at the 48-h time point, at which, amongst the small number of embryos, there were significantly more males than females in the 16-h compared with the 24-h IVM group ($P < 0.05$; Table 3).

Within each treatment, the observed sex ratio differed from the expected 1 : 1 at specific time points. For the 24-h IVM group there was a trend towards more males at the early cleaving time

Table 2. Effect of duration of *in vitro* maturation of bovine oocytes on blastocyst development

Eight replicates. Data are reported as *n* (% (mean ± s.e.m.)) for each day and IVM duration

Duration of IVM	No. of COC	Blastocyst yield		
		Day 6	Day 7	Day 8
16 h	1078	164 (17.1 ± 3.1)	314 (30.6 ± 4.1)	356 (34.1 ± 3.9)
24 h	1074	182 (16.4 ± 2.1)	376 (34.6 ± 3.5)	426 (39.4 ± 4.3)

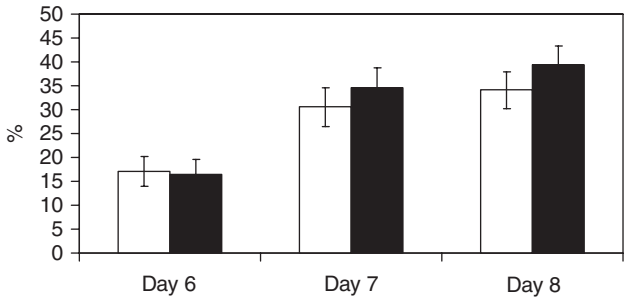


Fig. 2. Kinetics of blastocyst development *in vitro* following oocyte maturation for 16 (□, $n = 1078$) or 24 h (■, $n = 1074$) (8 replicates, $P > 0.05$).

points (up to and including 36 hpi) with a significant difference only at 32 hpi (59.7 *v.* 40.3%, $P < 0.05$) while at the later time points (40, 44 and 48 hpi) there was a tendency towards more females. For the 16-h IVM group there were significantly more males at 24, 28 and 32 hpi (100 *v.* 0%; 64.5 *v.* 35.5% and 60.1 *v.* 39.9% respectively, $P < 0.05$), and this trend continued at the later time points, although the difference was not significant (Table 3).

Irrespective of duration of IVM, when embryos were grouped into early- (up to and including 32 hpi) and late-cleaving (after 32 hpi) groups, there were significantly more males in the early group than the expected 1 : 1 (Table 4). However, in the 16-h IVM group the difference continued, in favour of males, in the late-cleaving embryos (Table 4). Analysis using logistic regression (sex regressed on IVM duration and time) supported this observation; there was no significant interaction between IVM and time but time significantly affected sex ratio ($P = 0.011$) while the effect of duration of IVM approached significance ($P = 0.097$).

The overall sex ratio of all 2-cell embryos at 48 hpi differed significantly from 1 : 1 in favour of males in both groups (24 h: 55.9 *v.* 44.1%; 16 h: 59.1 *v.* 40.9%, $P < 0.05$) (Table 4). The overall sex ratio of blastocysts differed significantly from 1 : 1 in both groups (24 h: 59.7 *v.* 40.3%; 16 h: 58.5 *v.* 41.5%, $P < 0.05$; Table 5). There was a tendency for more male blastocysts than females at Days 6, 7 and 8. However, a significant difference in favour of males compared with 1 : 1 was only apparent for the 24-h IVM group on Day 6 (62.0 *v.* 38.0%) and for the 16-h IVM group only on Day 7 (60.6 *v.* 39.4%).

Table 3. Effect of duration of *in vitro* oocyte maturation and timing of first cleavage on the sex ratio of bovine 2-cell embryos

Data are reported as *n* (%)

Duration of IVM	Sex ratio													
	24 h		28 h		32 h		36 h		40 h		44 h		48 h	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
24 h	6 (66.7)	3 (33.3)	86 (56.6)	66 (43.4)	108^{x} (59.7)	73^{y} (40.3)	39 (54.2)	33 (45.8)	14 (46.7)	16 (53.3)	8 (44.4)	10 (56.6)	1 (14.9)	6^{a} (85.1)
16 h	5^{x} (100.0)	0^{y} (0.0)	40^{x} (64.5)	22^{y} (35.5)	95^{x} (60.1)	63^{y} (39.9)	76 (55.9)	60 (44.1)	43 (59.7)	29 (40.3)	17 (50.0)	17 (50.0)	10 (58.8)	7^{b} (41.2)

^{a,b}Indicates a significant difference ($P < 0.05$) between treatments in sex ratio at a given time point.

^{x,y}Indicates a significant difference ($P < 0.05$) from the expected 1 : 1 ratio at a particular time point within each treatment group.

Table 4. Sex ratio of early- and late-cleaving bovine embryos following *in vitro* oocyte maturation for 16 or 24 h
Data are reported as *n* (%)

Duration of IVM	Sex ratio					
	Before 32 h		After 32 h		Total at 48 h	
	♂	♀	♂	♀	♂	♀
24 h	200^{x} (58.5)	142^{y} (41.5)	62 (48.8)	65 (51.2)	262^{x} (55.9)	207^{y} (44.1)
16 h	140^{x} (62.2)	85^{y} (37.8)	146^{x} (56.4)	113^{y} (43.6)	286^{x} (59.1)	198^{y} (40.9)

^{x,y}Indicates a significant difference ($P < 0.05$) from the expected 1 : 1 ratio within each treatment group.

Table 5. Sex ratio of blastocysts derived from oocytes matured for 16 or 24 h
Data are reported as *n* (%)

Duration of IVM	Blastocyst sex ratio							
	Day 6		Day 7		Day 8		Total	
	♂	♀	♂	♀	♂	♀	♂	♀
24 h	70^{x} (62.0)	43^{y} (38.0)	64 (58.2)	46 (41.8)	17 (56.7)	13 (43.3)	151^{x} (59.7)	102^{y} (40.3)
16 h	41 (52.6)	37 (47.4)	57^{x} (60.6)	37^{y} (39.4)	22 (66.7)	11 (33.3)	120^{x} (58.5)	85^{y} (41.5)

^{x,y}Indicates a significant difference ($P < 0.05$) from the expected 1 : 1 ratio within each treatment group.

Discussion

In the present study 4402 oocytes were used over 16 replicates to address the effect of the duration of IVM on the kinetics of cleavage, overall cleavage rate and blastocyst development. To address the effect of duration of IVM on the sex of embryos after IVF we sexed a total of 953 2-cell embryos and 458 blastocysts. The findings demonstrate that reducing the period of maturation *in vitro* before exposure to sperm from the standard 24 h to 16 h results in a slower evolution of cleavage after IVF and a slightly reduced cleavage rate overall. However, this delay in cleavage was not manifested in a lower blastocyst yield. Irrespective of the duration of IVM, early-cleaving embryos were more likely to be male.

A clear relationship between the time of first cleavage after insemination *in vitro* and the developmental competence of the embryo has been demonstrated in humans (Shoukir *et al.* 1997; Bos-Mikich *et al.* 2001; Fenwick *et al.* 2002) and in many domestic species, including cattle (Lonergan *et al.* 1999), with those oocytes cleaving earliest after IVF being more likely to reach the blastocyst stage than their later-cleaving counterparts. The mechanisms responsible for this difference have not been fully elucidated but it is related to the polyadenylation of several mRNA transcripts in the oocyte (Brevini *et al.* 2002). In addition, the sire used in IVF can have a significant effect on the kinetics of cleavage and the proportion of oocytes developing to the blastocyst stage (Ward *et al.* 2001). However, irrespective of sire, those zygotes that cleave earliest after insemination are more likely to form blastocysts than their later-cleaving counterparts. The mechanism through which the sire can affect the kinetics of cleavage is thought to be due to the timing of the onset and duration of DNA replication during the first cell cycle (Eid *et al.* 1994; Comizzoli *et al.* 2003). Using semen from the same bull, in the present study we observed that it is possible to influence the kinetics of oocyte cleavage by altering the maturational stage at which fertilisation takes place; IVM for 16 h resulted in a lag in cleavage kinetics compared with the 24-h IVM group.

From our results we can conclude also that even though during meiosis in mammals equal numbers of X- and Y-bearing sperm are created, this might not be reflected in the proportion of the sperm population that reaches the oocyte. The overall sex ratio of all 2-cell embryos at 48 hpi differed significantly from 1 : 1 in favour of males in both groups (144 males to 100 females at 16 h and 127 males to 100 females at 24 h). This is in agreement with the primary sex ratio (male : female ratio at the time of fertilisation) reported in humans, that may be as high as 170 males to 100 females (Pergament *et al.* 2002), suggesting that there is a differential ability of X- or Y-bearing spermatozoa to fertilise oocytes. These differences may be due to differences in the physiological activity (motility/viability or capacitation/acrosome reaction) of X- or Y-bearing spermatozoa before fertilisation (Penfold *et al.* 1998; Gutiérrez-Adán *et al.* 1999; Madrid-Bury *et al.* 2003).

In cattle, male embryos have been reported to develop to more advanced embryonic stages faster than females (Avery *et al.* 1991; Xu *et al.* 1992). Consistent with this, the proportion of males is significantly higher among embryos that cleave to the two-cell stage within the first 30 h of insemination (Yadav *et al.* 1993). When this is coupled with the fact that such oocytes account for most of blastocysts (Lonergan *et al.* 1999) it is not surprising that the sex ratio is skewed amongst blastocysts in many *in vitro* systems and the disproportionate number of male calves born after IVF, perhaps

by selecting developmentally-advanced blastocysts for transfer (Hasler 1998).

Both Dominko and First (1997) and Gutiérrez-Adán *et al*. (1999) found that insemination of oocytes immediately after polar body extrusion resulted in more female embryos, whereas delaying insemination for 8 h resulted in more males. They suggested that the observed skewing of the sex ratio away from the expected 1 : 1 was influenced by the maturational state of the oocytes and the kinetics of sperm–oocyte interaction. They propose that delaying insemination allows metaphase II-arrested oocytes to process Y sperm more effectively and that delaying insemination allows a greater proportion of oocytes to reach optimal maturity before insemination. Whether this is true or not is unclear. However, one reason for the difference in our results may be the fact that we did not select oocytes for the presence of a polar body.

Undoubtedly, a lower proportion of such unselected oocytes inseminated at 16 h are at metaphase II. We have previously shown that by 12 h IVM over 90% of oocytes have reached metaphase I; by 16 h over 50% are at either telophase I or metaphase II while by 24 h ~90% are at metaphase II (Lonergan *et al.* 1997). Thus in the present study approx 50% of oocytes inseminated at 16 h were at metaphase I (40%) or earlier stages. Similar to the present study, Agung *et al*. (2006) did not preselect oocytes based on polar body extrusion. Based on a small number of blastocysts sexed per time point ($n = 22$) in that study, the proportion of males increased with increasing duration of IVM from 32% in blastocysts derived from oocytes matured for 16 h, to 73% in oocytes matured for 34 h. In agreement with our observations, they did not observe a difference in blastocyst sex ratio following IVM for 16 *v.* 22 h.

The postfertilisation culture environment to which the bovine embryo is exposed has been shown to influence the quality of the resulting embryo measured in terms of cryotolerance (Rizos *et al.* 2002*a*), ultrastructural morphology (Rizos *et al.* 2002*b*), transcript abundance (Rizos *et al.* 2002*c*; Lonergan *et al.* 2003), incidence of chromosome abnormalities (Lonergan *et al.* 2004) and pregnancy rate (Lazzari *et al.* 2002). In addition, under certain conditions, particularly in the presence of serum, the speed of development (Rizos *et al.* 2003) and the sex ratio (Gutiérrez-Adán *et al.* 2001*b*) can be altered. In the present study, irrespective of treatment, the overall sex ratio at the 2-cell stage and the blastocyst stage were similar, suggesting that, at least under the conditions used in the present study, culture environment did not preferentially support the development of one sex over another.

In conclusion, timing of gamete interaction and maturity of the oocyte at the time of the interaction can affect the kinetics of the early cleavage divisions but has no effect on the sex ratio of the embryos produced.

Acknowledgements

This work was supported by the grants, AGL2006–05616 and AT2006–003 to Dr Dimitrios Rizos and AGL2006–04799 to Dr Alfonso Gutiérrez-Adán, from the Spanish Ministry of Science and Technology. Pablo Bermejo-Álvarez was supported by a FPU grant from the Spanish Ministry of Education and Science. Dr Patrick Lonergan is funded by Science Foundation Ireland.

References

Agung, B., Otoi, T., Wongsrikeao, P., Taniguchi, M., Shimizu, R., Watari, H., and Nagai, T. (2006). Effect of maturation culture period of oocytes on the sex ratio of *in vitro*-fertilized bovine embryos. *J. Reprod. Dev.* **52**, 123–127. doi:10.1262/JRD.17055

Avery, B., Madison, V., and Schmidt, M. (1991). Sex and development in bovine *in vitro*-fertilized embryos. *Theriogenology* **35**, 953–963. doi:10.1016/0093-691X(91)90306-X

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., and Gutierrez-Adan, A. (2008). Epigenetic differences between male and female bovine blastocysts produced *in vitro*. *Physiol. Genomics* **32**, 264–272.

Bos-Mikich, A., Mattos, A. L., and Ferrari, A. N. (2001). Early cleavage of human embryos: an effective method for predicting successful IVF/ICSI outcome. *Hum. Reprod.* **16**, 2658–2661. doi:10.1093/HUMREP/16.12.2658

Brevini, T. A., Lonergan, P., Cillo, F., Francisci, C., Favetta, L. A., Fair, T., and Gandolfi, F. (2002). Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol. Reprod. Dev.* **63**, 510–517. doi:10.1002/MRD.10191

Comizzoli, P., Urner, F., Sakkas, D., and Renard, J. P. (2003). Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the S phase in bovine zygotes. *Biol. Reprod.* **68**, 1934–1940. doi:10.1095/BIOLREPROD.102.011452

Dominko, T., and First, N. L. (1997). Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. *Theriogenology* **47**, 1041–1050. doi:10.1016/S0093-691X(97)00061-7

Eid, L. N., Lorton, S. P., and Parrish, J. J. (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biol. Reprod.* **51**, 1232–1237. doi:10.1095/BIOLREPROD51.6.1232

Fenwick, J., Platteau, P., Murdoch, A. P., and Herbert, M. (2002). Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. *Hum. Reprod.* **17**, 407–412. doi:10.1093/HUMREP/17.2.407

Garner, D. L., and Seidel, G. E., Jr (2008). History of commercializing sexed semen for cattle. *Theriogenology* **69**, 886–895. doi:10.1016/J.THERIOGENOLOGY.2008.01.006

Gutiérrez-Adán, A., Behboodi, E., Andersen, G. B., Medrano, J. F., and Murray, J. D. (1996). Relationship between stage of development and sex of bovine IVM-IVF embryos cultured *in vitro* versus in the sheep oviduct. *Theriogenology* **46**, 515–525. doi:10.1016/0093-691X(96)00173-2

Gutiérrez-Adán, A., Perez, G., Granados, J., Garde, J. J., Perez-Guzman, M., Pintado, B., and De La Fuente, J. (1999). Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. *Zygote* **7**, 37–43. doi:10.1017/S0967199499000374

Gutiérrez-Adán, A., Granados, J., Pintado, B., and De La Fuente, J. (2001*a*). Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured *in vitro*. *Reprod. Fertil. Dev.* **13**, 361–365. doi:10.1071/RD00039

Gutiérrez-Adán, A., Lonergan, P., Rizos, D., Ward, F. A., Boland, M. P., Pintado, B., and de la Fuente, J. (2001*b*). Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. *Theriogenology* **55**, 1117–1126. doi:10.1016/S0093-691X(01)00471-X

Hasler, J. F. (1998). The current status of oocyte recovery, *in vitro* embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. *J. Anim. Sci.* **76**, 52–74.

Jobst, S. M., and Nebel, R. L. (1998). Does timing of insemination affect gender of the resultant calf? *J. Dairy Sci.* **81**, 244. [Abstract]

Kochhar, H. S., Kochhar, K. P., Basrur, P. K., and King, W. A. (2003). Influence of the duration of gamete interaction on cleavage, growth rate and

sex distribution of *in vitro*-produced bovine embryos. *Anim. Reprod. Sci.* **77**, 33–49. doi:10.1016/S0378-4320(03)00006-X

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H., and Galli, C. (2002). Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biol. Reprod.* **67**, 767–775. doi:10.1095/BIOLREPROD.102.004481

Lonergan, P., Khatir, H., Carolan, C., and Mermillod, P. (1997). Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h. *J. Reprod. Fertil.* **109**, 355–365.

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., and Boland, M. P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* **117**, 159–167.

Lonergan, P., Rizos, D., Gutierrez-Adan, A., Moreira, P. M., Pintado, B., and Boland, M. P. (2003). Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or *in vivo*. *Biol. Reprod.* **69**, 1424–1431. doi:10.1095/BIOLREPROD.103.018168

Lonergan, P., Gervi-Pedersen, H., Rizos, D., Greve, T., Thomsen, P. D., Fair, T., Evans, A., and Boland, M. P. (2004). Effect of the post-fertilization culture environment on the incidence of chromosome aberrations in bovine blastocysts. *Biol. Reprod.* **71**, 1096–1100. doi:10.1095/BIOLREPROD.104.030635

Madrid-Bury, N., Fernández, R., Jiménez, A., Pérez-Garnelo, S., Moreira, P. N., Pintado, B., de la Fuente, J., and Gutiérrez-Adán, A. (2003). Effect of ejaculate, bull, and a double swim-up sperm processing method on sperm sex ratio. *Zygote* **11**, 229–235. doi:10.1017/S0967199403002272

Manna, L., Neglia, G., Marino, M., Gasparrini, B., Di Palo, R., and Zicarelli, L. (2003). Sex determination of buffalo embryos (*Bubalus bubalis*) by polymerase chain reaction. *Zygote* **11**, 17–22. doi:10.1017/S0967199403001035

Martinez, F., Kaabi, M., Martinez-Pastor, F., Alvarez, M., Anel, E., Boixo, J. C., de Paz, P., and Anel, L. (2004). Effect of the interval between estrus onset and artificial insemination on sex ratio and fertility in cattle: a field study. *Theriogenology* **62**, 1264–1270. doi:10.1016/J.THERIOGENOLOGY.2004.01.002

Penfold, L. M., Holt, C., Holt, W. V., Welch, G. R., Cran, D. G., and Johnson, L. A. (1998). Comparative motility of X and Y chromosome-bearing bovine sperm separated on the basis of DNA content by flow sorting. *Mol. Reprod. Dev.* **50**, 323–327. doi:10.1002/(SICI)1098-2795(199807)50:3<323::AID-MRD8>3.0.CO;2-L

Pergament, E., Toydemir, P. B., and Fiddler, M. (2002). Sex ratio: a biological perspective of 'Sex and the City'. *Reprod. Biomed. Online* **5**, 43–46.

Pursley, J. R., Silcox, R. W., and Wiltbank, M. C. (1998). Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. *J. Dairy Sci.* **81**, 2139–2144.

Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002*a*). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248. doi:10.1002/MRD.1153

Rizos, D., Fair, T., Papadopoulos, S., Boland, M. P., and Lonergan, P. (2002*b*). Developmental, qualitative and ultrastructural differences between ovine and bovine embryos produced *in vivo* or *in vitro*. *Mol. Reprod. Dev.* **62**, 320–327. doi:10.1002/MRD.10138

Rizos, D., Lonergan, P., Boland, M. P., Arroyo-Garcia, R., Pintado, B., de la Fuente, J., and Gutierrez-Adan, A. (2002*c*). Analysis of differential mRNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol. Reprod.* **66**, 589–595. doi:10.1095/BIOLREPROD66.3.589

Rizos, D., Gutiérrez-Adán, A., Pérez-Garnelo, S., de la Fuente, J., Boland, M. P., and Lonergan, P. (2003). Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biol. Reprod.* **68**, 236–243. doi:10.1095/BIOLREPROD.102.007799

Roelofs, J. B., Bouwman, E. B., Pedersen, H. G., Rasmussen, Z. R., Soede, N. M., Thomsen, P. D., and Kemp, B. (2006). Effect of time of artificial insemination on embryo sex ratio in dairy cattle. *Anim. Reprod. Sci.* **93**, 366–371. doi:10.1016/J.ANIREPROSCI.2005.09.004

Rorie, R. W. (1999). Effect of timing of artificial insemination on sex ratio. *Theriogenology* **52**, 1273–1280. doi:10.1016/S0093-691X(99)00216-2

Rorie, R. W., Lester, T. D., Lindsey, B. R., and McNew, R. W. (1999). Effect of timing of artificial insemination on gender ratio in beef cattle. *Theriogenology* **52**, 1035–1041. doi:10.1016/S0093-691X(99)00192-2

Seidel, G. E., Jr (2003). Sexing mammalian sperm – intertwining of commerce, technology, and biology. *Anim. Reprod. Sci.* **79**, 145–156. doi:10.1016/S0378-4320(03)00162-3

Seidel, G. E., Jr (2007). Overview of sexing sperm. *Theriogenology* **68**, 443–446. doi:10.1016/J.THERIOGENOLOGY.2007.04.005

Shoukir, Y., Campana, A., Farley, T., and Sakkas, D. (1997). Early cleavage of *in vitro*-fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Hum. Reprod.* **12**, 1531–1536. doi:10.1093/HUMREP/12.7.1531

Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., and Lonergan, P. (2001). Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. *Mol. Reprod. Dev.* **60**, 47–55. doi:10.1002/MRD.1060

Wehner, G. R., Wood, C., Tague, A., Barker, D., and Hubert, H. (1997). Efficiency of the OVATEC unit for estrus detection and calf sex control in beef cows. *Anim. Reprod. Sci.* **46**, 27–34. doi:10.1016/S0378-4320(96)01604-1

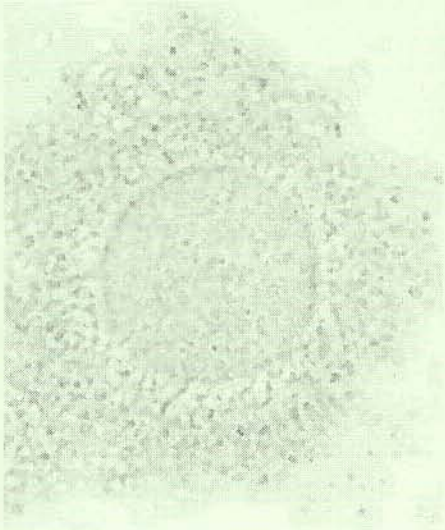
Xu, K. P., Yadav, B. R., King, W. A., and Betteridge, K. G. (1992). Sex-related differences in developmental rates of bovine embryos produced and cultured *in vitro*. *Mol. Reprod. Dev.* **31**, 249–252. doi:10.1002/MRD.1080310404

Yadav, B. R., King, W. A., and Betteridge, K. J. (1993). Relationships between the completion of first cleavage and the chromosomal complement, sex and developmental rates of bovine embryos generated *in vitro*. *Mol. Reprod. Dev.* **36**, 434–439. doi:10.1002/MRD.1080360405

Manuscript received 24 April 2008, accepted 2 June 2008

CHAPTER 2

CAN BOVINE IN VITRO MATURED OOCYTES SELECTIVELY PROCESS X- OR Y-SORTED SPERM DIFFERENTIALLY?



BIOLOGY OF REPRODUCTION **79,** 594–597 (2008)
Published online before print 25 June 2008.
DOI 10.1095/biolreprod.108.070169

Can Bovine In Vitro-Matured Oocytes Selectively Process X- or Y-Sorted Sperm Differentially?[1]

P. Bermejo-Álvarez,[3] D. Rizos,[3] D. Rath,[4] P. Lonergan,[4,5] and A. Gutiérrez-Adán[2,3]

Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos,[3] INIA, 28040 Madrid, Spain
Department of Biotechnology,[4] Institute of Animal Breeding (FAL), 31535 Neustadt, Germany
School of Agriculture, Food Science and Veterinary Medicine,[5] University College Dublin, Dublin 4, Ireland

ABSTRACT

It has been reported that the mammalian female could have a preconceptual influence on the sex of her offspring, and it has been hypothesized that this influence could go some way toward accounting for the reported lower fertility following insemination with sex-sorted sperm. To test whether in vitro matured oocytes are able to select X- or Y-bearing spermatozoa following in vitro fertilization (IVF), we fertilized in vitro 1788 oocytes with X-sorted semen, Y-sorted semen, a mix of X- and Y-sorted semen, and unsorted semen from the same bull, and cultured until Day 9. Fertility was assessed by recording cleavage rate at 48 h postinsemination (hpi) and blastocyst development until Day 9. Embryos were sexed at the two- to four-cell stage and the blastocyst stage. The proportion of zygotes cleaving at 48 hpi was not different between X- and Y-sorted groups and the mix of X- and Y-sorted semen group; however, all were significantly lower than the unsorted group ($P < 0.001$). Blastocyst yield on Day 6 was significantly higher ($P \leq 0.01$) in the control group compared with the rest of the groups. Cumulative blastocyst yields on Days 7, 8, and 9 were also significantly higher ($P \leq 0.01$) in the unsorted group compared with the sorted groups. The proportion of female and male two- to four-cell embryos obtained following IVF with X- and Y-sorted sperm was 88% and 89%, respectively and the sex ratio at the two- to four-cell stage was not different following IVF with unsorted or sorted/recombined sperm (56.9% males vs. 57% males, respectively). At the blastocyst stage, similar percentages were obtained. In conclusion, the differences in cleavage and blastocyst development using sorted versus unsorted sperm are not due to the oocyte preferentially selecting sperm of one sex over another, but are more likely due to spermatic damage caused by the sorting procedure.

animal reproduction technology, fertilization, in vitro fertilization, male and female bovine embryos, sex-sorted sperm

INTRODUCTION

Several factors have been reported to influence sex ratio in cattle, including 1) timing of insemination in vivo [1–3], 2) the maturational state of the oocyte at the time of insemination in vitro [4–6], 3) the duration of gamete coincubation in vitro [7], and 4) the postfertilization culture conditions in vitro [8]. It has been reported that the mammalian female could have a preconceptual influence on the sex of her offspring, and it has been hypothesized that this influence could go some way toward accounting for the reported lower fertility following insemination with sex-sorted sperm [9]. It has been suggested that the level of testosterone in bovine follicular fluid (FF) may predispose the ovulated oocyte to be preferentially fertilized by either an X- or a Y-chromosome-bearing spermatozoa [10]. In support of this theory, a positive correlation has been described between the concentration of testosterone in follicular fluid and the likelihood of the oocyte recovered from that follicle being fertilized in vitro by a Y-bearing spermatozoa [11]. If this notion is true, then no matter how technically successful sperm sorting techniques are, fertility rates will be low.

Lower pregnancy rates from sex-sorted sperm have been attributed to factors associated with the sorting process (sperm damage), problems with insemination techniques, and/or the low sperm doses typically used. Experiments using matched doses of sorted and unsorted sperm attempt to address the issue of sperm numbers used [12]. However, the comparison between insemination with sorted versus sorted/recombined sperm, which would control for both sperm dose and sperm damage, has not been made.

Therefore, this study was designed to address two questions: 1) Can in vitro matured oocytes preferentially select X- or Y-bearing spermatozoa following in vitro fertilization (IVF)? and 2) Is there a difference in fertility following insemination with sorted versus sorted/recombined sperm? To do this, we inseminated in vitro matured oocytes with X-sorted semen, Y-sorted semen, a mix of X- and Y- sorted semen (i.e., sorted-recombined) or unsorted semen from the same high-fertility bull. Fertility was assessed by recording cleavage rate at 48 h postinsemination (hpi) and blastocyst development until Day 9. Embryos were sexed at the two- to four-cell stage and the blastocyst stage.

MATERIALS AND METHODS

Semen Preparation

Semen was collected from six ejaculates of a Holstein Friesian bull of proven fertility and diluted immediately with Sexcess extender (Masterrind, Verden Germany) to a final concentration of 1×10^8 sperm/ml. Spermatozoa were labelled with 15–25 μl of a 8.12 mM Hoechst 33342 solution (8.9 mM of 2-[-4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2.5-bi-1H-benzimidazole in bidistillated water) for 60 min at 34°C. Sperm sorting was performed (one sorting per ejaculate) according to the Beltsville Sperm Sorting Technology [13]. Labeled sperm samples were filtered through a 51-μm cell strainer grid (Falcon Becton Dickinson and Company, Franklin Lakes, NY) and then supplemented with 1 μl food dye solution FD&C#40 (Warner Jekinson Company Inc., St. Louis, MO). Sorting was performed with a high-speed flow cytometer (MoFlo SX, DakoCytomation, Fort Collins, CO), equipped with an

[1]Supported by the Grants, AGL2006-04799 to A.G.-A., and AGL2006-05616 and AT2006-003 to D. Rizos from the Spanish Ministry of Science and Technology. P.B.-Á. was supported by a FPU grant from the Spanish Ministry of Education and Science. P.L. is funded by Science Foundation Ireland.
[2]Correspondence: Alfonso Gutiérrez-Adán, Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain. FAX: 034 91 347 4014; e-mail: agutierr@inia.es

Received: 22 April 2008.
First decision: 10 May 2008.
Accepted: 6 June 2008.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Effect on cleavage rate and blastocyst development (four replicates) from using sex-sorted bovine sperm in IVF.

No. of oocytes	Percentage cleaved (n)	Percentage of blastocysts (n)			
		Day 6	Day 7	Day 8	Day 9
X-sorted					
518	61.1 ± 3.2[b] (313)	6.9 ± 2.4[b] (33)	21.0 ± 4.1[b] (106)	25.6 ± 4.4[b] (130)	27.0 ± 4.8[b] (137)
Y-sorted					
445	61.3 ± 5.7[b] (277)	8.2 ± 2.1[b] (36)	22.1 ± 4.3[b] (99)	25.6 ± 4.4[b] (116)	27.3 ± 4.6[b] (123)
XY pool[a]					
400	58.5 ± 5.4[b] (235)	5.3 ± 2.3[b] (21)	17.1 ± 4.3[b] (70)	21.1 ± 4.8[b] (85)	22.6 ± 4.6[b] (91)
Unsorted					
425	84.4 ± 1.7[c] (358)	22.3 ± 5.8[c] (90)	40.7 ± 3.8[c] (169)	48.9 ± 5.0[c] (202)	50.4 ± 5.3[c] (208)

[a] Produced by pooling an equal volume of Percoll-separated X- and Y-sorted sperm.
[b,c] Different superscripts indicate significant differences between groups ($P < 0.01$) based on ANOVA.

argon UV-Laser (Coherent Laser, Inova I 90C-6, Dieburg, Germany), set to 200 mW output. Samples were sorted at an average event rate of 25 000 cells/sec giving a sorting rate of 3300 cells/sec. Spermatozoa were collected into 10-ml conical plastic tubes (Greiner, Nürtingen, Germany) prefilled with 500 μl TEST-yolk extender [13]. Immediately after collection of 8 million spermatozoa, the sorted cells were centrifuged at 840 × *g* for 20 min. The supernatant was discharged, and the pellet was resuspended with a TRIS-based cooling extender and cooled in two steps to 4°C within 3 h. Then the final sperm concentration was set at 20.6×10^6 sperm/ml with a TRIS based freezing extender [14], and 3.3×10^6 spermatozoa were filled into 0.25-ml plastic straws (Minitüb, Tiefenbach, Germany), sealed, and frozen in liquid nitrogen. From each sorted frozen sample, a purity analysis for the correct sex separation was performed using a flow-cytometrical resort protocol and by curve-fitting statistics (Gaus 7 [13]). Semen collection was carried out under routine conditions with due adherence to the care and welfare of the animal. All the procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of INIA (Madrid) and were performed in accordance with the guiding principles for the care and use of laboratory animals.

In Vitro Embryo Production

Immature cumulus oocyte complexes (COCs; n = 1788) were obtained by aspirating follicles (2–8 mm) from the ovaries of heifers and cows collected at slaughter. COCs were matured (in groups of approximately 50 in 500 μl of medium) for 24 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. For IVF, matured COCs were inseminated with 12.5 μl of frozen-thawed, percoll-separated sperm added to 25-μl droplets under mineral oil (15–20 oocytes per droplet) at a final concentration of 1×10^6 spermatozoa/ml. Oocytes were inseminated with frozen-thawed X-sorted sperm (n = 518), Y-sorted sperm (n = 445), a pool of X- and Y-sorted sperm (n = 400), or unsorted sperm (n = 425) from the same bull. To generate the pool of X- and Y-sorted sperm, an equal volume of X-sorted and Y-sorted sperm were mixed after percoll separation. Theoretically, this pooled XY group should yield the same sex ratio as unsorted sperm. Four replicates (= days of ovary collection) were carried out, and each treatment was represented in each replicate; approximately equal numbers of oocytes were used per treatment in each replicate. Gametes were coincubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 hpi, presumptive zygotes were denuded and transferred to 25-μl culture droplets (one embryo per μl) under mineral oil. Culture took place in SOF + 5% FCS. Plates were incubated for 7 days at 39°C under an atmosphere of 5% CO_2, 90% of N_2, and 5% O_2 with maximum humidity. Fertility was assessed by recording cleavage rate at 48 hpi and blastocyst development until Day 9. Embryos were sexed at the two- to four-cell stage and the blastocyst stage.

Embryo Sexing by PCR

For sexing, embryos were washed in PBS and transferred into 5 mg/ml pronase (Sigma, Madrid, Spain) in PBS for 1 min to remove the zona pellucida and any attached spermatozoa. This was considered necessary to avoid any potential ambiguity in the sexing results contributed by accessory sperm attached to the zona. They were then washed three to four times in PBS, individually snap frozen in liquid nitrogen in 0.2-μl Eppendorf tubes, and stored at −80°C until analysis. For increasing PCR efficiency, embryos were digested with 8-μl/individual two- to four-cell embryo or 16-μl/individual blastocyst of a 100-μg/ml proteinase K (Sigma, P8044-1G) solution at 55°C overnight. After digestion, proteinase K was inactivated at 95°C for 10 min. Two sets of PCR primers were used to determine embryo sex: Y-chromosome specific primers (BRY1a), and bovine specific satellite sequence primers (Sat1) [15]. The PCR reactions were conducted in a total volume of 25 μl containing 8 μl of the proteinase K digested sample, 1× Gotaq Flexi buffer, 1 IU of Gotaq (Promega, Madison, WI), 1.25 mM $MgCl_2$, 0.1 mM dNTP, 1 ng/μl BRY primers, and 0.2 ng/μl Sat primers. PCR was performed with a first cycle (94°C for 3 min, 60°C for 40 sec, and 72°C for 15 sec) followed by 35 cycles (94°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec; final elongation 72°C for 5 min) [16]. Products were visualized on an ethidium bromide stained 2% agarose gel. The gel was visualized under ultraviolet illumination for the positive 300-bp band of BRY 1a and 216 bp of the satellite sequence. Samples that exhibited both bands were assigned as male, while samples exhibiting only a satellite sequence band were assigned as female. Every PCR was carried out with three controls: male genomic DNA, female genomic DNA, and a negative control.

Statistical Analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. Cleavage and embryo development was analyzed using one-way repeated measures ANOVA with arcsine transformation.

RESULTS

In Vitro Development of Bovine Embryos Produced Using Sorted Sperm

Cleavage was assessed at 48 hpi, and blastocyst yield was recorded on Days 6, 7, 8, and 9. The proportion of zygotes cleaving at 48 hpi was not different between X- and Y-sorted groups and the mix of X- and Y-sorted semen group (X: 61.1 ± 3.2%, Y: 61.3 ± 5.7%, XY: 58.5 ± 5.4%); however, all were significantly lower than the unsorted group (84.2 ± 1.3%; $P < 0.001$) (Table 1). Blastocyst yield (% out of total COCs) on Day 6 was significantly higher ($P \leq 0.01$) in the unsorted control group compared with the rest of the groups (unsorted: 22.3 ± 5.8; X: 6.9 ± 2.42; Y: 8.2 ± 2.1; XY: 5.3 ± 2.4). Similarly, cumulative blastocyst yields on Days 7, 8, and 9 were significantly higher ($P \leq 0.01$) in the unsorted group compared with the sorted groups (e.g., Day 9, unsorted: 50.4 ± 5.3; X: 27.0 ± 4.8; Y: 27.3 ± 4.6; XY: 22.6 ± 4.6) (Table 1). Thus, reconstituting an "unsorted" sperm sample by recombining X- and Y-sorted sperm did not improve cleavage rate or blastocyst yield compared with the use of X- or Y-sorted sperm.

Sex Ratio of Bovine Embryos Produced Using Sorted and Unsorted Sperm

The proportion of female and male two- to four-cell embryos obtained following IVF with X- and Y-sorted sperm

TABLE 2. Effect on the proportion of male and female 2- to 4-cell embryos at 48 hpi and blastocysts on Day 8 (four replicates) from using sex-sorted bovine sperm in IVF.

Sperm group	No. of 2- to 4-cell embryos sexed	Percentage male (n)	Percentage female (n)	No. of blastocysts sexed	Percentage male (n)	Percentage female (n)
X-sorted	183	12.0 (22)	88.0 (161)	123	12.2 (15)	87.8 (108)
Y-sorted	163	89.0 (145)	11.0 (18)	112	89.3 (100)	10.7 (12)
XY pool[a]	86	57.0 (49)	43.0 (37)	79	50.6 (40)	49.4 (39)
Unsorted	109	56.9 (62)	43.1 (47)	157	53.5 (84)	46.5 (73)

[a] Produced by pooling an equal volume of Percoll-separated X- and Y-sorted sperm.

was 88% and 89%, respectively (Table 2). The sex ratio at the two- to four-cell stage was not different following IVF with unsorted or sorted/recombined sperm (56.9% males vs. 57% males, respectively; Table 2).

The sex ratio at the blastocyst stage was consistent with that observed at 48 hpi; the proportion of female and male embryos obtained with X- and Y-sorted sperm was 87.8% and 89.3%, respectively (Table 2), while the sex ratio was not different between unsorted or sorted/recombined sperm (53.5% vs. 50.6%, respectively; Table 2). This observed consistency between sex ratio at the two- to four-cell stage and the blastocyst stage would suggest that the postfertilization conditions of culture used in this study did not preferentially support the development of embryos of either sex.

DISCUSSION

It has been suggested that in cattle the oocyte is ovulated already adapted to receive an X- or Y-bearing spermatozoa, and this could be responsible for some of the fertilization failure observed when using sex-sorted semen [9]. We have found that when in vitro matured bovine oocytes were fertilized with X- or Y-sorted sperm or with a reconstituted mixture of X- or Y-sorted sperm, embryo development was similar, indicating that bovine oocytes, at least under the conditions used in this study, cannot preferentially select X- or Y-bearing spermatozoa. Moreover, in agreement with previous reports, we have found that the production of embryos using IVF with sorted sperm is lower than using nonsorted sperm. The evidence suggests that this is due to damage induced during the sorting process as the cells are exposed to a number of potential hazards, including dilution, centrifugation, incubation, exposure to DNA stains, high pressure, laser light, and electrical charge [17]. Several studies have reported effects of the sorting procedure on sperm, including altered motility patterns [18], a reduced life span [19], acceleration of the acrosome reaction [20], and increases in the proportion of capacitated sperm [21]. Changes in viability/motility and capacitation/acrosome reaction of sex-sorted sperm could be the reason for the reduced initial fertilization rates obtained in vitro and in vivo. Moreover, it has been also reported that sperm chromosome integrity and sperm DNA fragmentation are not affected by the sorting procedure and may even be improved in the sorted population because of the elimination of membrane damaged sperm in the sorting process [22, 23]. In addition, the quality of the embryos produced using sex-sorted spermatozoa may be affected; differential expression of developmentally important genes in embryos derived from unsorted and sex-sorted sperm has been reported [24].

Several different maternal and paternal traits may affect offspring sex ratio in mammals. The results of a recent meta-analysis suggested that sex ratio adjustment in mammals was most likely to occur around conception and/or before implantation [25], and the two most significant factors affecting the sex ratio variation was the time of insemination and the condition of the mother around conception [25]. It has been reported that variation in glucose levels, in tandem with other mechanisms, may mediate the sex of an offspring [26–28]. Indirect evidence supporting this hypothesis comes from studies showing that female mice fed with a high-fat diet, leading potentially to increased glucose levels [29], produced male-biased litters [30]. There is also increasing interest in hormones as proximate mechanisms of offspring sex ratio variation. It has been reported that maternal testosterone level during fertilization may influence offspring sex ratio [31]. Recently, it has been suggested that high follicular testosterone concentration in cattle is associated with male embryos after fertilization [11]. On the other hand, females may produce male-biased litters when mated with a highly fertile male [32], of which high testosterone level can be a signal [33]. The variation in glucose levels may be related to the maternal hormone levels because glucose levels are important for reproductive functioning through their interaction with LH. Glucose enhances LH secretion and release from the pituitary, and reduced glucose concentration can inhibit LH pulses [34]. Moreover, LH has an enhancing effect on glycolytic activity, increasing glucose availability to the oocyte [35]. Also, it has been recently reported in voles that the proportion of male pups was positively associated with levels of maternal circulating testosterone and glucose just prior to breeding [30]. Taken together, these results suggest that several different maternal and paternal traits may affect offspring sex ratio in mammals.

In conclusion, our results strongly suggest that the differences in cleavage and blastocyst development using sorted versus unsorted sperm are not due to the oocyte preferentially selecting sperm of one sex over another, as has been recently suggested [9], but more likely are due to sperm damage caused by the sorting procedure.

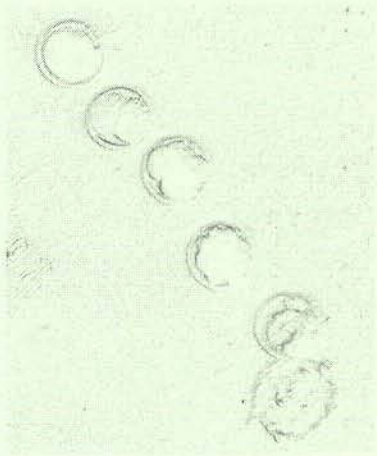
REFERENCES

1. Wehner GR, Wood C, Tague A, Barker D, Hubert H. Efficiency of the OVATEC unit for estrus detection and calf sex control in beef cows. Anim Reprod Sci 1997; 46:27–34.
2. Pursley JR, Silcox RW, Wiltbank MC. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J Dairy Sci 1998; 81:2139–2144.
3. Martinez F, Kaabi M, Martinez-Pastor F, Alvarez M, Anel E, Boixo JC, de Paz P, Anel L. Effect of the interval between estrus onset and artificial insemination on sex ratio and fertility in cattle: a field study. Theriogenology 2004; 62:1264–1270.
4. Dominko T, First NL. Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. Theriogenology 1997; 47:1041–1050.
5. Gutierrez-Adan A, Perez G, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. Zygote 1999; 7:37–43.
6. Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, Nagai T. Effect of maturation culture period of oocytes on the sex ratio of in vitro fertilized bovine embryos. J Reprod Dev 2006; 52:123–127.
7. Kochhar HS, Kochhar KP, Basrur PK, King WA. Influence of the duration

of gamete interaction on cleavage, growth rate and sex distribution of in vitro produced bovine embryos. Anim Reprod Sci 2003; 77:33–49.
8. Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, de la Fuente J. Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 2001; 55:1117–1126.
9. Grant VJ, Chamley LW. Sex-sorted sperm and fertility: an alternative view. Biol Reprod 2007; 76:184–188.
10. Grant VJ, Irwin RJ. Follicular fluid steroid levels and subsequent sex of bovine embryos. J Exp Zoolog A Comp Exp Biol 2005; 303:1120–1125.
11. Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. Biol Reprod 2008; 78:812–815.
12. Seidel GE Jr, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD, Cran DG. Insemination of heifers with sexed sperm. Theriogenology 1999; 52:1407–1420.
13. Johnson LA, Welch GR, Rens W. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci 1999; 77(suppl 2):213–220.
14. Klinc P, Rath D. Reduction of oxidative stress in bovine spermatozoa during flow cytometric sorting. Reprod Domest Anim 2007; 42:63–67.
15. Manna L, Neglia G, Marino M, Gasparrini B, Di Palo R, Zicarelli L. Sex determination of buffalo embryos (*Bubalus bubalis*) by polymerase chain reaction. Zygote 2003; 11:17–22.
16. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiol Genomics 2008; 32:264–272.
17. Maxwell WM, Evans G, Hollinshead FK, Bathgate R, De Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK. Integration of sperm sexing technology into the ART toolbox. Anim Reprod Sci 2004; 82–83: 79–95.
18. Suh TK, Schenk JL, Seidel GE Jr. High pressure flow cytometric sorting damages sperm. Theriogenology 2005; 64:1035–1048.
19. Hollinshead FK, Gillan L, O'Brien JK, Evans G, Maxwell WM. In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. Reprod Fertil Dev 2003; 15: 351–359.
20. Moce E, Graham JK, Schenk JL. Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. Theriogenology 2006; 66:929–936.
21. Maxwell WM, Long CR, Johnson LA, Dobrinsky JR, Welch GR. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 1998; 10:433–440.
22. Boe-Hansen GB, Morris ID, Ersboll AK, Greve T, Christensen P. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. Theriogenology 2005; 63:1789–1802.
23. De Ambrogi M, Spinaci M, Galeati G, Tamanini C. Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology 2006; 66:1994–2000.
24. Morton KM, Herrmann D, Sieg B, Struckmann C, Maxwell WM, Rath D, Evans G, Lucas-Hahn A, Niemann H, Wrenzycki C. Altered mRNA expression patterns in bovine blastocysts after fertilisation in vitro using flow-cytometrically sex-sorted sperm. Mol Reprod Dev 2007; 74:931–940.
25. Cameron EZ. Facultative adjustment of mammalian sex ratios in support of the Trivers-Willard hypothesis: evidence for a mechanism. Proc Biol Sci 2004; 271:1723–1728.
26. Cameron EZ, Lemons PR, Bateman PW, Bennett NC. Experimental alteration of litter sex ratios in a mammal. Proc Biol Sci 2008; 275:323–327.
27. Gutierrez-Adan A, Granados J, Pintado B, De La Fuente J. Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. Reprod Fertil Dev 2001; 13:361–365.
28. Jimenez A, Madrid-Bury N, Fernandez R, Perez-Garnelo S, Moreira P, Pintado B, de la Fuente J, Gutierrez-Adan A. Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. Mol Reprod Dev 2003; 65:180–187.
29. Folmer V, Soares JC, Gabriel D, Rocha JB. A high fat diet inhibits delta-aminolevulinate dehydratase and increases lipid peroxidation in mice (*Mus musculus*). J Nutr 2003; 133:2165–2170.
30. Rosenfeld CS, Grimm KM, Livingston KA, Brokman AM, Lamberson WE, Roberts RM. Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. Proc Natl Acad Sci U S A 2003; 100:4628–4632.
31. James WH. Testosterone levels, handedness and sex ratio at birth. J Theor Biol 1988; 133:261–266.
32. Gomendio M, Malo AF, Soler AJ, Fernandez-Santos MR, Esteso MC, Garcia AJ, Roldan ER, Garde J. Male fertility and sex ratio at birth in red deer. Science 2006; 314:1445–1447.
33. Mills SC, Grapputo A, Koskela E, Mappes T. Quantitative measure of sexual selection with respect to the operational sex ratio: a comparison of selection indices. Proc Biol Sci 2007; 274:143–150.
34. Murahashi K, Bucholtz DC, Nagatani S, Tsukahara S, Tsukamura H, Foster DL, Maeda KI. Suppression of luteinizing hormone pulses by restriction of glucose availability is mediated by sensors in the brain stem. Endocrinology 1996; 137:1171–1176.
35. Zuelke KA, Brackett BG. Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. Endocrinology 1992; 131:2690–2696.

CHAPTER 3

DEVELOPMENTAL KINETICS AND GENE EXPRESSION IN MALE AND FEMALE EMBRYOS PRODUCED *IN VITRO* WITH SEX-SORTED SPERMATOZOA



CSIRO PUBLISHING

Reproduction, Fertility and Development, 2010, **22**, 426–436 www.publish.csiro.au/journals/rfd

Developmental kinetics and gene expression in male and female bovine embryos produced *in vitro* with sex-sorted spermatozoa

Pablo Bermejo-Álvarez[A], *Patrick Lonergan*[B], *Detlef Rath*[C], *Alfonso Gutiérrez-Adan*[A] *and Dimitrios Rizos*[A,D]

[A]Departamento de Reproducción Animal y Conservaciónde Recursos Zoogenéticos, INIA, Carretera De la Coruña Km 5.9, Madrid 28040, Spain.

[B]School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

[C]Department of Biotechnology, Institute of Animal Breeding (FAL), Mariensee, Neustadt 31535, Germany.

[D]Corresponding author. Email: drizos@inia.es

Abstract. Using bovine embryos generated *in vitro* from IVF with X-sorted, Y-sorted and unsorted spermatozoa, we compared the kinetics of male and female embryo development and gene expression between male and female blastocysts. Bovine *in vitro*-matured oocytes ($n = 8858$) were fertilised with spermatozoa from each of three different bulls (X-sorted, Y-sorted or unsorted spermatozoa depending on the experiment). The cleavage rate was assessed 24, 27, 30, 33, 36, 40, 44 and 48 h post insemination (h.p.i.) and blastocyst development was recorded on Days 6–9. The relative mRNA abundance of nine genes (*GSTM3*, *DNTM3A*, *PGRMC1*, *TP53*, *BAX*, *COX2*, *IGF2R*, *AKR1B1* and *PLAC8*) was analysed in male and female Day 7 blastocysts produced with sorted and unsorted spermatozoa from one bull. Cumulative cleavage rate and blastocyst yield were significantly higher in the unsorted group compared with the X- or Y-sorted group from the same bull ($P \leq 0.05$). Although differences existed between bulls in terms of cleavage rate, no differences were observed in cleavage rate between X- and Y-sorted spermatozoa within a bull. The blastocyst yield was significantly higher only for Bull 3 when the Y-sorted spermatozoa were used ($27.1 + 2.8$ *v.* $19.1 + 1.4$ for Y- and X-sorted spermatozoa, respectively; $P < 0.05$). There were no differences in the mRNA abundance of the nine genes analysed between embryos of the same sex produced with sorted or unsorted spermatozoa. However, significant differences in polyA mRNA abundance were observed between male and female blastocysts for three genes (*GSTM3*, *DNMT3A* and *PGRMC1*; $P \leq 0.05$). In conclusion, the use of sorted rather than unsorted spermatozoa in IVF significantly delays the onset of first cleavage. Differences were noted between bulls, but not between X- and Y-sorted spermatozoa, and although no differences were found in terms of the mRNA abundance of the nine genes tested between sorted and unsorted spermatozoa, sex-related differences were found in the case of three genes.

Additional keyword: IVF.

Introduction

Together with sex determination by embryo biopsy, sperm sexing by flow cytometry currently represents the only reliable method by which to obtain offspring of a predetermined sex. Over the past decade, procedures for sexing mammalian spermatozoa by flow cytometry/cell sorting have been refined sufficiently for large-scale commercial application in cattle, with millions of doses of sexed spermatozoa being sold each year for artificial insemination (Seidel 2009). In human assisted reproductive technology (ART), sperm sorting is an emerging tool to reduce the risk of X-linked genetic disorders or for family balancing (Karabinus 2009).

As well as the obvious benefits to commercial animal production, the availability of sex-sorted spermatozoa has enormous benefits for the basic study of early embryo development in a sex-specific manner. Because at present the main use of sexed spermatozoa is for the production of replacement heifers in the dairy industry, most spermatozoa currently marketed are X-sorted only. Indeed, there are very few publications where the consequences of insemination with X- or Y-sorted spermatozoa from the same bull have been compared (Morton *et al*. 2007; Bermejo-Alvarez *et al*. 2008*b*).

Following IVF, the speed of development, specifically the timing of the first cleavage division and/or the timing of the appearance of the blastocyst, has been related to embryo developmental competence (Lonergan *et al*. 1999) and has been reported to be altered when using sorted spermatozoa both *in vivo* (McNutt and Johnson 1996) and *in vitro* (Cran *et al*. 1993;

 10.1071/RD09142 1031-3613/10/020426

Lu *et al*. 1999). Furthermore, under some culture conditions, male embryos have been found to develop faster and are thought to be of better quality than their female counterparts (Avery *et al*. 1991; Xu *et al*. 1992).

Following insemination with sex-sorted spermatozoa, lower fertility rates have been observed both *in vivo* (Schenk *et al*. 1999; Bodmer *et al*. 2005) and *in vitro* (Lu *et al*. 1999; Bermejo-Alvarez *et al*. 2008*b*; Xu *et al*. 2009). Several factors have been proposed for the reduced fertility rates, including the lower doses of spermatozoa used (Bodmer *et al*. 2005), damage to the spermatozoa caused by the sorting procedure (Schenk and Seidel 2007) and differences in individual sires (for a review, see Rath *et al*. 2009). In addition, it has been proposed that some oocytes may be predisposed to being fertilised by spermatozoa of a particular sex (Grant *et al*. 2008), although this has recently been questioned (Bermejo-Alvarez *et al*. 2008*b*). In a recent large field trial using sexed spermatozoa from seven Holstein bulls, Frijters *et al*. (2009) reported a 13.6% decline in the 56-day non-return rate; approximately two-thirds of this decline (8.6%) was attributed to the low dose used and one-third (5%) was attributed to the sorting process itself.

On the available evidence, the offspring produced by sex-sorted spermatozoa do not show an increased frequency of abnormalities (Tubman *et al*. 2004). However, during the sorting process, spermatozoa are exposed to several potential hazards (Maxwell *et al*. 2004) that have been suggested to be responsible for some deleterious effects, such as altered motility patterns (Suh *et al*. 2005), reduced lifespan (Hollinshead *et al*. 2003), acceleration of the acrosome reaction (Mocé *et al*. 2006) and increases in the proportion of capacitated spermatozoa (Maxwell *et al*. 1998). Lower fertility and/or embryo quality may be expected as a consequence of these changes. It has been suggested that damaged spermatozoa may be able to fertilise an oocyte, which results in low-quality embryos (Fernández-Gonzalez *et al*. 2008). Some studies have reported abnormalities in terms of mRNA abundance or ultrastructure in embryos produced by sorted spermatozoa (Morton *et al*. 2007; Palma *et al*. 2008), whereas others have found no differences in the percentage of transferable or degenerate embryos or pregnancy rates between embryos produced with sorted and unsorted spermatozoa (Hayakawa *et al*. 2009). In addition, sex-related differences have been reported in terms of embryo metabolism (Tiffin *et al*. 1991), survival after vitrification (Nedambale *et al*. 2004), mRNA abundance (Gutiérrez-Adán *et al*. 2000; Kobayashi *et al*. 2006; Morton *et al*. 2007; Bermejo-Alvarez *et al*. 2008*a*) and epigenetic status (Bermejo-Alvarez *et al*. 2008*a*).

Thus, in the present study, we used X- and Y-sorted spermatozoa from each of three sires and almost 9000 immature oocytes to address the following questions: (*1*) is the lower blastocyst development observed *in vitro* when using sex-sorted spermatozoa related to a different pattern of cleavage kinetics after IVF; (*2*) is the pattern of cleavage of oocytes different between male and female embryos produced following IVF with X-sorted *v.* Y-sorted spermatozoa; (*3*) is there a difference in the relative transcript abundance of developmental-related genes between blastocysts derived from sorted *v.* unsorted spermatozoa that could be due to damage to the spermatozoa sustained during the sorting procedure; and (*4*) does the relative transcript abundance of the same transcripts differ between male and female blastocysts produced with sorted or unsorted spermatozoa?

Materials and methods

Collection and sorting of spermatozoa

Semen was collected from three Holstein Friesian bulls of proven fertility and diluted immediately with Sexcess extender (Masterrind, Verden, Germany) to a concentration of 1×10^8 spermatozoa mL^{-1}. Spermatozoa were labelled with 15–25 μL of an 8.12 mM Hoechst 33342 solution (8.9 mM 2-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole in double-distilled water) for 60 min at 34°C. Sperm sorting was performed (one sorting per ejaculate) according to the Beltsville Sperm Sorting Technology (Johnson *et al*. 1999). Labelled sperm samples were filtered through a 51-μm cell strainer grid (Falcon Becton Dickinson, Franklin Lakes, NY, USA) and then supplemented with 1 μL food dye solution FDandC#40 (Warner Jekinson, St Louis, MO, USA). Sorting was performed at 40 psi with a high-speed flow cytometer (MoFlo SX; DakoCytomation, Fort Collins, CO, USA) equipped with an argon UV-Laser (Inova I 90C-6; Coherent Laser, Dieburg, Germany) set to 200 mW output. Samples were sorted at an average event rate of 25 000 cells s^{-1}, giving a sorting rate of 3300 cells s^{-1}. Spermatozoa were collected into 10-mL conical plastic tubes (Greiner, Nürtingen, Germany) prefilled with 500 μL Test-yolk extender (Johnson *et al*. 1999). Immediately after collection of 8 million spermatozoa, sorted cells were centrifuged at 840*g* for 20 min. The supernatant was discarded and the pellet was resuspended with a TRIS-based cooling extender and cooled to 4°C within 2 h. Then, the final concentration was set 20.6×10^6 spermatozoa mL^{-1} with a TRIS-based freezing extender (Klinc and Rath 2007) and 0.25-mL plastic straws (Minitüb, Tiefenbach, Germany) were filled with 3.3×10^6 spermatozoa (Segment 1 of the straws was filled with 160 μL sperm and Segment 2 was filled with 50 μL extender), sealed and frozen in liquid nitrogen. From each sorted frozen sample, a purity analysis for the correct sex separation was performed using a flow cytometrical resort protocol and by curve fitting statistics (Gaus 7; Johnson *et al*. 1999). Semen collection was performed under routine conditions with due adherence to the care and welfare of the animal.

In vitro embryo production

As described previously (Rizos *et al*. 2002), immature cumulus–oocyte complexes (COCs; $n = 8858$, 22 replicates) were obtained by aspirating follicles (2–8 mm) from the ovaries of heifers and cows collected at slaughter. The COCs were matured for 24 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng mL^{-1} epidermal growth factor at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. The IVF procedure was performed with X- or Y-sorted spermatozoa from three different bulls or with unsorted or a pool of X- and Y-sorted spermatozoa from one of the bulls (Bull 1). To generate the pool of X- and Y-sorted spermatozoa, an equal volume of X-sorted and Y-sorted spermatozoa was mixed after Percoll separation. Matured COCs were inseminated with 12.5 μL

frozen–thawed, Percoll-separated semen added to 25-μL droplets under mineral oil (15–20 oocytes per droplet) at a final concentration of 10^6 spermatozoa mL^{-1}. Gametes were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination (h.p.i.), presumptive zygotes were denuded and transferred to 25-μL culture droplets (1 embryo per μL) under mineral oil. Culture took place in synthetic oviducal fluid (SOF) + 5% fetal calf serum (FCS). Plates were incubated for 8 days at 39°C under an atmosphere of 5% CO_2, 90% N_2 and 5% O_2 with maximum humidity. Fertility was assessed by recording cleavage rates at 24, 27, 30, 33, 36, 40, 44 and 48 h.p.i. and blastocyst development until Day 9.

Embryo sexing by polymerase chain reaction

For Experiment 1, sexing was performed as described by Bermejo-Alvarez *et al*. (2008*b*). Two- to four-cell embryos were washed in phosphate-buffered saline (PBS) and transferred into 5 mg mL^{-1} pronase (Sigma, Madrid, Spain) in PBS for 1 min to remove the zona pellucida and any attached spermatozoa. This was considered necessary to avoid any potential ambiguity in the sexing results as a result of accessory spermatozoa attached to the zona. The embryos were then washed three to four times in PBS, snap frozen individually in liquid nitrogen in 0.2-mL Eppendorf tubes and stored at −80°C until analysis. To increase polymerase chain reaction (PCR) efficiency, embryos were digested with 8 μL of 100 μg mL^{-1} proteinase K (P8044–1G; Sigma) at 55°C overnight. After digestion, proteinase K was inactivated at 95°C for 10 min. Two sets of PCR primers were used to determine embryo sex: Y-chromosome-specific primers (*BRY1A*) and bovine-specific satellite sequence primers (*SAT1*; Manna *et al*. 2003). The PCR reactions were performed in a total volume of 25 μL containing 8 μL proteinase K-digested sample, 1× Gotaq Flexi buffer (Promega, Madison, WI, USA), 1 IU of Gotaq (Promega, Madison, WI, USA), 1.25 mM $MgCl_2$, 0.1 mM dNTP, 1 ng μL^{-1} BRY primers and 0.2 ng μL^{-1} Sat primers. The PCR was performed with one cycle of 94°C for 3 min, 60°C for 40 s and 72°C for 15 s, followed by 35 cycles or 94°C for 15 s, 60°C for 30 s and 72°C for 15 s with final elongation at 72°C for 5 min (Bermejo-Alvarez *et al*. 2008*a*). Products were visualised on an ethidium bromide-stained 2% agarose gel. The gel was visualised under ultraviolet light for the positive 300-bp band of *BRY1A* and the 216-bp band of the satellite sequence. Samples that exhibited both bands were designated male, whereas samples exhibiting only a satellite sequence band were designated female. Each PCR was performed with three controls: male genomic DNA, female genomic DNA and a negative control.

RNA and DNA extraction, reverse transcription and quantification of mRNA transcript abundance

Poly(A) RNA was extracted from individual blastocysts using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions but with some minor modifications. After 5 min incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet, suspended in Washing Buffer A and stored at 4°C while DNA extraction and sexing was performing. The DNA present in Lysis Buffer was extracted with phenol/chloroform treatment and finally suspended in 16 μL MilliQ water; an 8-μL aliquot of each sample was used to perform embryo sexing by PCR under the same conditions as described above. After embryo sexing, the individually stored poly(A) RNA from 10 embryos of the same sex was pooled and RNA extraction continued. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers and MMLV reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were were then incubated at 42°C for 60 min to allow the reverse transcription of RNA, followed by incubation at 70°C for 10 min to denature the RT enzyme.

The quantification of all mRNA transcripts was performed by real-time quantitative (q) RT-PCR. For qRT-PCR, five groups of cDNA per experimental group, each obtained from 10 embryos, were used with two repetitions for all genes of interest. Experiments were conducted to compare relative levels of each transcript with those of histone H2AFZ in each sample. The PCR was performed by adding a 2-μL aliquot of each sample to the PCR mix containing specific primers to amplify histone H2Az (*H2AFZ*), glutathione-S-transferase Mu3 (*GSTM3*), DNA methyltransferase 3α (*DNMT3A*), progesterone receptor membrane component 1 (*PGRMC1*), tumour protein 53 (*TP53*), Bcl-2-associated X protein (*BAX*), prostaglandin G/H synthase-2 (*COX2*), insulin-like growth factor 2 receptor (*IGF2R*), aldo-keto reductase family 1 member B1 (*AKR1B1*) and placenta-specific 8 (*PLAC8*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Table 1. For quantification, real-time PCR was performed as described above. The comparative cycle threshold (CT) method was used to quantify expression levels (Bermejo-Alvarez *et al*. 2008*a*). Quantification was normalised against that of the endogenous control, *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the *H2AFZ* CT value for each sample from the CT value for each gene in the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target gene were determined using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Data were analysed using SigmaStat (Jandel Scientific, San Rafael, CA, USA). Cleavage, embryo development and mRNA expression were analysed using one-way repeated-measures ANOVA with arcsine transformation.

Table 1. Details of primers used for quantitative reverse transcription–polymerase chain reaction

Gene	Primer sequence (5′–3′) forward and reverse	Fragment size (bp)	GenBank accession no.
H2AFZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
GSTM3	ATCGCCCGCAAGCACAATATG TCCAGGCACTTGGGCTCAAAC	290	NM_001046560
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	317	AY271299
PGRMC1	TGTGTGTCACAAATCCAGAAAG AATCATGCAGTTAGGTCAATCG	265	NM_001075133.1
TP53	CTCAGTCCTCTGCCATACTA GGATCCAGGATAAGGTGAGC	364	U74486
BAX	CTACTTTGCCAGCAAACTGG TCCCAAAGTAGGAGAGGA	158	NM_173894.1
COX2	ATCTACCCGCCTCATGTTCCT GGATTAGCCTGCTTGTCTGGA	187	AF031698
IGF2R	GCTGCAGTGTGCCAAGTGAAAAAG AGCCCCTCTGCCATTGTTACCT	201	NM_174352.2
AKR1B1	CGTGATCCCCAAGTCAGTGA AATCCCTGTGGGAGGCACA	152	NM_001012519.1
PLAC8	CGGTGTTCCAGAGGTTTTTCC AAGATGCCAGTCTGCCAGTCA	166	NM_001025325.1

Experiment 1: validation of the sperm sorting procedure

The aim of this experiment was to validate the sex-sorting procedure by determining the sex of two- to four-cell embryos produced after IVF. *In vitro*-matured oocytes were inseminated with X- or Y-sorted spermatozoa from each of three bulls and the resulting zygotes were cultured *in vitro* as described above and sexed at the two- to four-cell stage at 48 h.p.i. ($n = 904$ embryos in total; three replicates per bull).

Experiment 2: effect of the sperm sorting procedure on cleavage and blastocyst development

The aim of this experiment was to examine the effect of sperm sorting on the speed of embryo development during the first 48 h after IVF and on blastocyst yield. Bovine COCs ($n = 2154$; five replicates) were matured *in vitro*, divided randomly into four groups and fertilised with unsorted, X-sorted, Y-sorted or a pool of X- and Y-sorted spermatozoa from the same bull (Bull 1). The resulting zygotes were cultured *in vitro*; the cleavage rate was assessed at 24, 27, 30, 33, 36, 40, 44 and 48 h.p.i. and blastocyst yield was recorded on Days 6, 7, 8 and 9 post insemination (p.i.).

Experiment 3: effect of sex on embryo cleavage and blastocyst development

The aim of this experiment was to compare the kinetics of the cleavage of male and female bovine embryos following IVF and subsequent blastocyst yield. To generate male and female embryos, bovine COCs ($n = 7855$) were matured *in vitro* and fertilised with X- or Y-sorted spermatozoa from one of three different bulls. The resulting zygotes were cultured *in vitro* and the kinetics of cleavage was assessed at 24, 27, 30, 33, 36, 40, 44 and 48 h.p.i. in a subset of COCs ($n = 2826$ COCs; 12 replicates), whereas total cleavage rate (at 48 h.p.i.) and blastocyst yield on Days 6, 7, 8 and 9 were recorded for all 7855 COCs (22 replicates).

Experiment 4: effect of sorting procedure and embryo sex on blastocyst mRNA abundance

This experiment was conducted to analyse differences in the relative transcript abundance of nine candidate genes (*GSTM3*, *DNMT3A*, *PGRMC1*, *TP53*, *BAX*, *COX2*, *IGFR2*, *AKR1B1* and *PLAC8*) between male and female blastocysts (i.e. the effect of embryo sex) produced with sorted (X and Y) and unsorted spermatozoa (i.e. the effect of the sorting procedure) from the same bull (Bull 1). Day 7 blastocysts derived from IVF with X-sorted, Y-sorted or unsorted sperm were snap frozen individually in liquid nitrogen following zona removal and stored at −80°C. Prior to pooling in groups of 10 embryos of the same sex for transcript analysis, each embryo was sexed as described above.

Results

Experiment 1: validation of the sperm sorting procedure

As shown in Table 2, approximately 90% of embryos produced following IVF with putative X- or Y-sorted spermatozoa from each of the three bulls were of the predicted sex, which is comparable results obtained in studies *in vivo* (Tubman *et al.* 2004).

Experiment 2: effect of the sperm sorting procedure on kinetics of cleavage and blastocyst development

The cumulative cleavage rate was significantly higher ($P \leq 0.001$) in the unsorted group compared with the three groups fertilised with sorted spermatozoa from 27 to 48 h.p.i. No differences were found between the group fertilised with

Table 2. Effect of using sex-sorted bovine spermatozoa in IVF on the proportion of male and female two- to four-cell embryos at 48 h post insemination (Experiment 1)

	No. embryos sexed	No. male embryos (%)	No. female embryos (%)
Bull 1			
X-sorted	183	22 (12.0)	161 (88.0)
Y-sorted	163	145 (89.0)	18 (11.0)
Bull 2			
X-sorted	132	7 (5.3)	125 (94.7)
Y-sorted	155	145 (93.5)	10 (6.5)
Bull 3			
X-sorted	142	15 (10.6)	127 (89.4)
Y-sorted	129	112 (86.8)	17 (13.2)
Total			
X-sorted	457	44 (9.6)	413 (90.4)
Y-sorted	447	402 (90.0)	45 (10.0)

X- or Y-sorted sperm or the reconstituted pool of X- and Y-sorted spermatozoa (Fig. 1*a*).

Furthermore, the progression of first cleavage clearly differed between the unsorted group, in which more than half of the total cleaved embryos had divided before 30 h.p.i. (i.e. $53.6 \pm 5.7\%$ cleaved at 30 h.p.i. of $84.2 \pm 1.3\%$ cleaved at 48 h.p.i.), and the sorted groups, in which, at the same time, only one-third of the total of cleaved embryos had cleaved (X-sorted: $20.3 \pm 3.2\%$ cleaved at 30 h.p.i. of $58.9 \pm 3.3\%$ cleaved at 48 h.p.i.; Y-sorted: $22.7 \pm 4.9\%$ cleaved at 30 h.p.i. of $62.2 \pm 4.5\%$ cleaved at 48 h.p.i.; pooled X- and Y-sorted: $14.1 \pm 4.4\%$ cleaved at 30 h.p.i. of $57.6 \pm 4.3\%$ cleaved at 48 h.p.i.; Fig. 1*b*).

Consistent with the observations regarding cleavage rate, blastocyst yield was significantly higher ($P \leq 0.05$) in the unsorted group compared with the sorted groups. However, a similar timing of blastocyst appearance was observed in all groups, with Days 6 and 7 accounting for most of the total blastocysts, irrespective of the treatment group (Fig. 1*c*).

Experiment 3: effect of X- or Y-sorted sperm on cleavage and blastocyst development

In terms of the kinetics of cleavage, no differences were observed between X- and Y-sorted spermatozoa at any time-point analysed (Fig. 2*a*).

To more clearly examine potential differences in the kinetics of embryo cleavage between different bulls, X- and Y-sorted data were separated (Fig. 2*b*). Differences between bulls appeared at specific time points and were similar for embryos of both sexes. Furthermore, clear differences in the progression of cleavage were present between the three bulls (Fig. 2*b*, *c*).

For a given bull, there was no difference in overall cleavage rate between X- and Y-sorted spermatozoa. However, significant differences existed in cleavage rate between X- or Y-sorted spermatozoa from different bulls (Table 3).

Cumulative blastocyst yield did not differ between bulls or between X- and Y-sorted spermatozoa for a given bull on Day 6 or 7 for Bulls 1 and 2. For Bull 3, a significantly higher blastocyst yield was obtained following insemination with Y- compared with X-sorted spermatozoa on Days 8 (25.5 ± 2.7 *v.* 18.3 ± 1.5, respectively; $P \leq 0.05$) and 9 (27.1 ± 2.8 *v.* 19.1 ± 1.4, respectively; $P \leq 0.05$; Table 3). For the X-sorted groups, no significant differences were observed between the three bulls. However, for the Y-sorted groups, Bull 2 showed a significantly higher blastocyst yield than Bulls 1 and 3 on Days 8 and 9 ($P \leq 0.05$). The speed of development was similar between bulls, with most blastocysts appearing on Day 7.

Experiment 4: effect of sorting procedure and embryo sex on blastocyst mRNA abundance

Sperm sorting had no effect on blastocyst transcript abundance; no differences were observed between blastocysts of the same sex produced with sorted or unsorted spermatozoa (Fig. 3). However, three genes displayed significant differences in transcript abundance between male and female blastocysts, irrespective of whether those blastocysts were produced with sorted or unsorted sperm ($P \leq 0.001$). The expression of *GSTM3* was fourfold higher in female compared with male embryos, whereas a 2.2-fold increase was observed in female compared with male blastocysts for *PGRMC1*. The opposite was observed for *DNMT3A*, with expression being 1.8-fold higher in male compared with female embryos. In addition, a tendency was noted for higher expression of *TP53* in male embryos ($P = 0.078$).

Discussion

When sorted spermatozoa are used in IVF, lower fertility rates are generally obtained compared with the use of unsorted spermatozoa (Lu *et al*. 1999; Bermejo-Alvarez *et al*. 2008*b*). This is generally attributed to the deleterious effect of the sex-sorting procedure on sperm capacitation status and lifespan (Maxwell *et al*. 2004). Although there is some evidence to suggest that embryos produced with sorted spermatozoa may develop slower than normal, both *in vivo* (McNutt and Johnson 1996) and *in vitro* (Cran *et al*. 1993; Lu *et al*. 1999), clear data describing the progression of embryo cleavage following insemination with sorted sperm are lacking.

In the present study, when sorted and unsorted spermatozoa from the same bull were used (in Experiment 2), we observed a significant reduction in the cleavage rate during the period when, under normal circumstances, most zygotes undergo the first mitotic division (from 27 to 32 h.p.i.; Ward *et al*. 2002). However, apart from this decrease in cleavage rate, we observed clear differences in the progression of cleavage; oocytes fertilised with sorted spermatozoa showed a slower early cleavage compared with those inseminated with unsorted spermatozoa. This slower cleavage may be associated with the reduced motility reported for sorted cryopreserved spermatozoa (Schenk *et al*. 1999; Hollinshead *et al*. 2003).

Male embryos produced using unsorted spermatozoa have been reported to develop faster than their female counterparts (Avery *et al*. 1991; Xu *et al*. 1992). The availability of X-sorted, Y-sorted and unsorted spermatozoa from the same sire allowed the rare opportunity to investigate potential differences in development between male and female embryos. Yadav *et al*. (1993) observed a higher proportion of males (77%) among the embryos

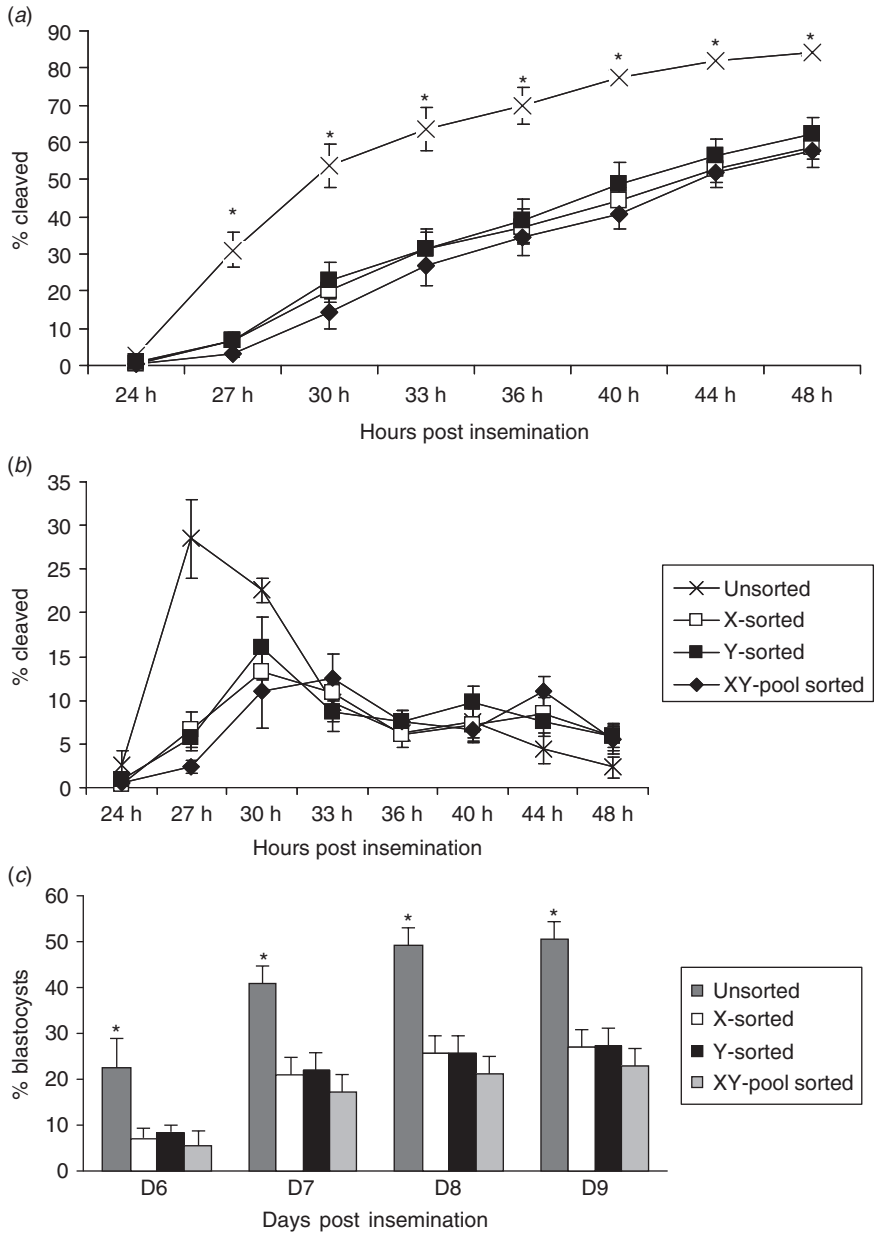


Fig. 1. Experiment 2. Kinetics of first cleavage division in bovine zygotes following insemination with unsorted ($n = 516$), X-sorted ($n = 618$), Y-sorted ($n = 533$) or pooled X- and Y-sorted ($n = 487$) spermatozoa from Bull 1 (five replicates). (*a*) Cumulative cleavage; (*b*) proportion of zygotes cleaving at each time point; (*c*) blastocyst cumulative yield. $^*P \leq 0.05$ between unsorted and sorted groups (ANOVA).

that cleaved to the two-cell stage before 30 h.p.i. after performing IVF with unsorted spermatozoa. Taking into account the fact that embryos that cleave before 30 h.p.i. account for most of the subsequent blastocysts (Lonergan *et al*. 1999), a skew in the sex ratio of blastocysts may be expected. Using sex-sorted spermatozoa from three different bulls (Experiment 3), we did not observe any significant differences in the kinetics of cleavage between male and female embryos produced with spermatozoa from the same bull. This is consistent with the results of a previous study (Rizos *et al*. 2008) that followed a different experimental design, performing IVF with unsorted spermatozoa and sexing two-cell embryos at different time-points after insemination. However, in that study, significantly more males cleaved before 32 h.p.i. (59.7% males), but the differences were not as impressive as previously reported (Yadav *et al*. 1993). This slightly higher proportion of males was also observed by using sorted and unsorted spermatozoa (57% and 56.9%, respectively) in another study (Bermejo-Alvarez *et al*. 2008*b*) and is consistent

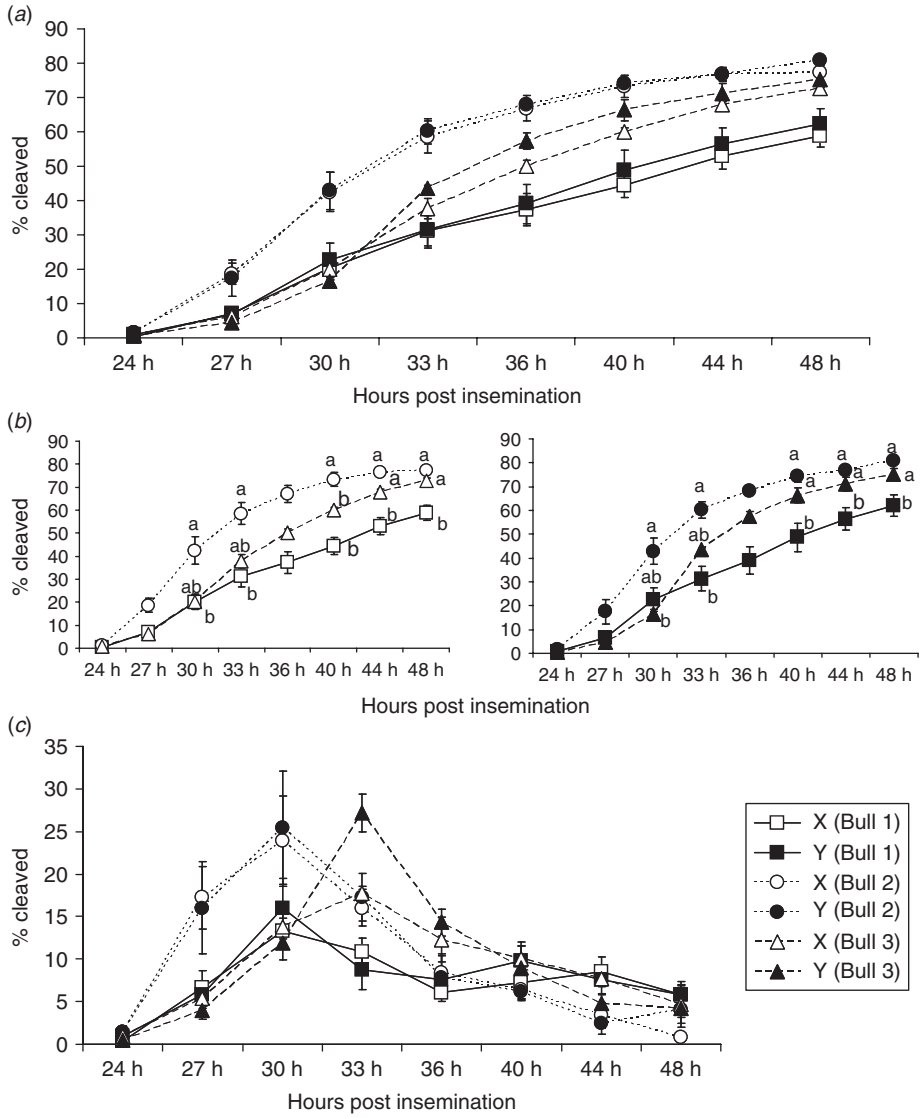


Fig. 2. Experiment 3. Kinetics of first cleavage division in bovine zygotes following insemination with X-sorted (closed symbols) or Y-sorted (open symbols) spermatozoa from each of three bulls: Bull 1 (squares; five replicates; X-sorted $n = 618$; Y-sorted $n = 533$), Bull 2 (diamonds; four replicates; X-sorted $n = 445$; Y-sorted $n = 411$) or Bull 3 (triangles; four replicates; X-sorted $n = 411$; Y-sorted $n = 408$). (*a*, *b*) Cumulative cleavage; (*c*) proportion of zygotes cleaving at each time-point. Middle graphs (*b*) compare different bulls when using X-sorted (left) or Y-sorted (right) spermatozoa; different letters indicate significant differences ($P \leq 0.05$) based on ANOVA.

with a non-significantly higher proportion of embryos cleaved after using Y- compared with X-sorted spermatozoa in all three bulls. This observation is in agreement with that reported by Beyhan *et al*. (1999) and is consistent with studies reporting a skew in the sex ratio in favour of males following IVF for both embryos (Yadav *et al*. 1993; Gutiérrez-Adán *et al*. 1996) and offspring (Lonergan *et al*. 1999; Luna *et al*. 2007). Together, these results suggest that the fertilisation capacity of Y-bearing spermatozoa following IVF may be slightly higher than that of X-bearing spermatozoa.

It has been suggested that embryos produced with sorted spermatozoa may be of inferior quality, based on reduced cell number (Beyhan *et al*. 1999), reduced timing of development (Cran *et al*. 1993; Lu *et al*. 1999) and differences in mRNA abundance (Morton *et al*. 2007). Our results showed clearly that the timing of development at the blastocyst stage was similar to that obtained using unsorted spermatozoa, with most blastocysts appearing on Day 7. To further investigate the effect of sperm sorting on embryo quality, we analysed the mRNA abundance of several candidate genes in blastocysts of the same sex produced with sorted or unsorted spermatozoa. A detailed analysis of the data according to sex was considered crucial, because gene expression between male and female embryos is known to differ (Gutiérrez-Adán *et al*. 2000; Kobayashi *et al*. 2006;

Table 3. Overall cleavage rate and blastocyst yield following insemination of *in vitro*-matured bovine oocytes with X- or Y-sorted spermatozoa using three bulls (Experiment 3)

For kinetics of cleavage, see Fig. 2. For X-sorted spermatozoa, there were nine, five and eight replicates for Bulls 1, 2 and 3, respectively (total = 22 replicates); for Y-sorted spermatozoa, there were eight, five and seven replicates for Bulls 1, 2 and 3, respectively (total = 20 replicates). Data are the mean ± s.e.m. [a,b]Different superscripts show significant differences ($P \leq 0.05$) between X- and Y-sorted spermatozoa from the same bull; [x,y,z]different superscripts show significant differences ($P \leq 0.05$) between X-sorted spermatozoa from different bulls; [e,f,g]different superscripts show significant differences ($P \leq 0.05$) between Y-sorted spermatozoa from different bulls

	No. oocytes	% Cleaved (*n*)	% Blastocysts (*n*)			
			Day 6	Day 7	Day 8	Day 9
Bull 1						
X-sorted	1782	62.2 ± 1.4^{z} (1115)	6.0 ± 1.1 (101)	17.1 ± 2.1 (279)	20.4 ± 2.5 (331)	21.2 ± 2.7 (343)
Y-sorted	1652	63.6 ± 2.4^{g} (1073)	6.9 ± 1.1 (103)	20.0 ± 2.2 (304)	24.3 ± 2.1^{f} (379)	25.5 ± 2.2^{f} (359)
Bull 2						
X-sorted	816	77.1 ± 1.8^{x} (611)	8.4 ± 1.8 (54)	21.5 ± 3.1 (163)	27.4 ± 3.2 (207)	28.4 ± 3.0 (213)
Y-sorted	756	80.5 ± 1.1^{e} (606)	9.6 ± 2.5 (57)	26.3 ± 3.2 (180)	35.6 ± 2.8^{e} (246)	36.8 ± 2.6^{e} (255)
Bull 3						
X-sorted	1575	70.3 ± 2.0^{y} (1077)	4.3 ± 1.1 (60)	13.7 ± 1.7 (202)	18.3 ± 1.5^{a} (270)	19.1 ± 1.4^{a} (284)
Y-sorted	1274	72.0 ± 2.0^{f} (896)	6.7 ± 1.4 (78)	18.4 ± 2.0 (209)	$25.5 \pm 2.7^{b,f}$ (298)	$27.1 \pm 2.8^{b,f}$ (317)

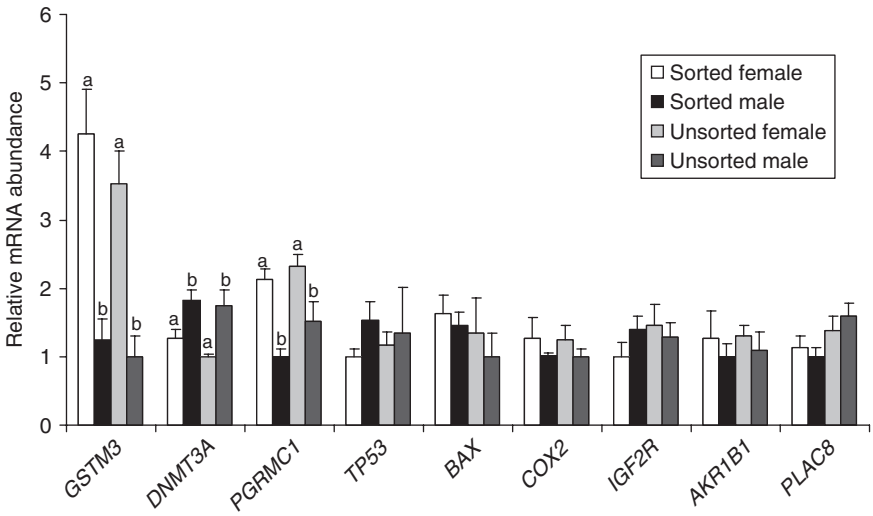


Fig. 3. Experiment 4. Relative mRNA expression of nine candidate genes related to the anti-oxidant response (*GSTM3*), DNA methylation (*DNMT3A*), pregnancy recognition and placental formation (*PGRMC1*, *COX2*, *IGF2R*, *AKR1B1* and *PLAC8*) and apoptosis (*TP53* and *BAX*). Different letters indicate significant differences ($P \leq 0.001$) based on ANOVA.

Morton *et al*. 2007; Bermejo-Alvarez *et al*. 2008*a*). In contrast with the report of Morton *et al*. (2007), we did not find any differences in gene expression between blastocysts of the same sex produced with sorted and unsorted spermatozoa in the present study. However, three genes differed significantly between male and female blastocysts, irrespective of whether they had been produced with sorted or unsorted spermatozoa.

Of the risks associated with the sperm sorting procedure, Hoechst 33342 has been reported to cause chromosomal damage under some conditions (Libbus *et al*. 1987). Sperm DNA damage has been correlated with pregnancy outcome (Benchaib *et al*. 2003; Virro *et al*. 2004), altered mRNA patterns and long-term effects in offspring (Fernández-Gonzalez *et al*. 2008). If this damage is not repaired by the oocyte, it could cause a pro-apoptotic profile in the resulting blastocyst. To test this hypothesis, the relative mRNA abundance of two genes related with apoptosis (i.e. *TP53* and *BAX*) and one gene involved in detoxification of ultraviolet-induced oxygen radicals (*GSTM3*; Strange *et al*. 1998) was analysed and no significant differences were found between embryos produced with sorted or unsorted spermatozoa. This observation is consistent with the lack of differences in levels of single- and double-stranded DNA breaks in embryos produced *in vitro* using sexed or non-sexed spermatozoa (Blondin *et al*. 2009). No significant damage to sperm chromatin structure was found after sorting (Suh *et al*. 2005) and no evidence of DNA nicks (Catt *et al*. 1997), ultraviolet-induced DNA damage (Catt *et al*. 1997) or changes to sperm chromatin structure has been obtained. As a result, DNA damage caused by the sorting procedure is estimated to be 3.2% (Garner 2006), which is much lower than the DNA fragmentation of the

spermatozoa analysed in previous studies (Benchaib *et al*. 2003; Fernández-Gonzalez *et al*. 2008) and it is likely to be repaired by the oocyte.

To determine whether a transcript abundance pattern reflective of high-quality embryos known to yield a pregnancy (El-Sayed *et al*. 2006) existed in embryos produced with sorted or unsorted spermatozoa, five genes related to pregnancy recognition and placental formation (i.e. *PGRMC1*, *COX2*, *AKR1B1*, *IGFR2* and *PLAC8*) were analysed. *PGRMC1* is a membrane-bound progesterone receptor that mediates anti-apoptotic effects of progesterone in granulosa cells (Peluso *et al*. 2006). Prostaglandin G/H synthase 2 (*COX2*) is related to prostaglandin synthesis and is more abundant in blastocysts resulting in calf delivery compared with those resulting in resorption (El-Sayed *et al*. 2006). The *AKR1B1* gene encodes the enzyme aldolase reductase, which metabolise progesterone and synthesises prostaglandin (PG) $F_{2\alpha}$, terminating pregnancy. The expression of *AKR1B1* has been associated with embryos that fail to establish pregnancies or are resorbed (El-Sayed *et al*. 2006). The *IGF2R* gene is an imprinted gene, the downregulation of which has been linked to fetal overgrowth (Young *et al*. 2001). *PLAC8* is an invasion-specific gene that is more abundant in blastocysts resulting in calf delivery compared with those resulting in resorption (El-Sayed *et al*. 2006). No significant differences in transcript abundance were observed between blastocysts produced with sorted or unsorted spermatozoa, which is in agreement with the observation that embryos obtained with sexed spermatozoa result in reasonable pregnancy rates that lead to term calves (Garner 2006; Xu *et al*. 2006). Finally, *DNMT3A* is a *de novo* methyltransferase involved in DNA methylation set up in early development, the expression of which has been reported to differ between male and female bovine *in vitro*-produced blastocysts (Bermejo-Alvarez *et al*. 2008*a*). No differences were found between embryos of the same sex produced with sorted or unsorted embryos. These results indicate that embryos produced either with sorted or unsorted spermatozoa show the same sex-specific patterns of gene expression. This important finding validates the use of sorted spermatozoa to generate large numbers of embryos of the desired sex to study preimplantation sexual dimorphism.

Three of the genes analysed in the present study, namely *GSTM3*, *DNMT3A* and *PGRMC1*, exhibited sex-related differences in expression. *DNMT3A* has been reported previously to be upregulated in male compared with female bovine blastocysts, which has been linked to sex-related epigenetic differences in terms of DNA methylation and telomere length (Bermejo-Alvarez *et al*. 2008*a*). *GSTM3* is involved in the detoxification of electrophilic compounds, such as oxygen radicals, by conjugation with glutathione. Because of this anti-oxidant action, *GSTM3* has been proposed as a DNA protector, because mutations and polymorphisms of this gene have been related with an increased risk of cancer, hypertension and Alzheimer's disease (Reszka *et al*. 2007). In mice treated with an anti-oxidant response inductor, *GSTM3* expression in the liver was sixfold higher in females than in males (Chanas *et al*. 2002). The anti-oxidant action of this gene could be related to differences in both metabolic and anti-oxidant responses reported between male and female blastocyst (for a review, see Gutiérrez-Adán *et al*. 2006). In addition, it has been reported that DNA hypermethylation regulates the expression of *GSTM3* (Peng *et al*. 2009), which provides a possible link between activation of the glutathione pathway and epigenetic status, because a higher methylation level has been reported in male blastocysts (Bermejo-Alvarez *et al*. 2008*a*). PGRMC1 is a progesterone membrane receptor that contains both high- and low-affinity progesterone binding site domains (Meyer *et al*. 1996); it is present in both the placenta and uterus during early pregnancy in the mouse (Zhang *et al*. 2008) and has been detected recently in bovine blastocysts (Clemente *et al*. 2009). The expression of PGRMC1 was found to be higher in female than male embryos in the present study. The biological significance of this difference is unclear, although a possible anti-apoptotic effect could be suggested, because PGRMC1 has been found to attenuate apoptosis in cultured rat granulosa cells in response to progesterone treatment (Peluso *et al*. 2006). Consistently, in the present study *TP53* expression tended to be higher in male embryos ($P \leq 0.078$). Studies *in vivo* have found that supplementary progesterone from Day 5 to Day 9 enhances both interferon-τ activity and trophoblast length in Day 16 bovine embryos (Garrett *et al*. 1988; Mann *et al*. 2006).

In conclusion, the main findings of the present study were that: (*1*) the use of sex-sorted spermatozoa was associated with a delay in the timing of the first cleavage following IVF, which is reflected in a lower blastocyst yield than that achieved using unsorted spermatozoa; (*2*) there are no significant differences in the kinetics of the first cleavage of embryos derived from X- and Y-sorted spermatozoa; (*3*) the kinetics of first cleavage differ between bulls; (*4*) there are no differences in the mRNA abundance of the nine genes analysed between blastocysts produced with sorted or unsorted spermatozoa, validating the use of sorted spermatozoa to generate a large number of embryos of the desired sex for development studies, as well as the use of sorted spermatozoa to obtain healthy offspring; and (*5*) there were sex-related differences in the relative transcript abundance of three genes.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Technology (AGL2006-05616 and AT2006-003 (INIA) to D.R.; and AGL2006-04799 to A.G.-A.). P.B.-A. was supported by an FPU grant from the Spanish Ministry of Education and Science. P.L. was funded by the Science Foundation Ireland.

References

Avery, B., Madison, V., and Greve, T. (1991). Sex and development in bovine *in-vitro* fertilized embryos. *Theriogenology* **35**, 953–963. doi:10.1016/0093-691X(91)90306-X

Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H., and Guerin, J. F. (2003). Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum. Reprod.* **18**, 1023–1028. doi:10.1093/HUMREP/DEG228

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., and Gutierrez-Adan, A. (2008*a*). Epigenetic differences between male and female bovine blastocysts produced *in vitro*. *Physiol. Genomics* **32**, 264–272. doi:10.1152/PHYSIOLGENOMICS.00234.2007

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., and Gutierrez-Adan, A. (2008*b*). Can bovine *in vitro*-matured oocytes selectively

process X- or Y-sorted sperm differentially? *Biol. Reprod.* **79**, 594–597. doi:10.1095/BIOLREPROD.108.070169

Beyhan, Z., Johnson, L. A., and First, N. L. (1999). Sexual dimorphism in IVM-IVF bovine embryos produced from X and Y chromosome-bearing spermatozoa sorted by high speed flow cytometry. *Theriogenology* **52**, 35–48. doi:10.1016/S0093-691X(99)00108-9

Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P., and King, W. A. (2009). Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* **71**, 30–38. doi:10.1016/J.THERIOGENOLOGY.2008.09.017

Bodmer, M., Janett, F., Hassig, M., den Daas, N., Reichert, P., and Thun, R. (2005). Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions. *Theriogenology* **64**, 1647–1655. doi:10.1016/J.THERIOGENOLOGY.2005.04.011

Catt, S. L., Sakkas, D., Bizzaro, D., Bianchi, P. G., Maxwell, W. M., and Evans, G. (1997). Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. *Mol. Hum. Reprod.* **3**, 821–825. doi:10.1093/MOLEHR/3.9.821

Chanas, S. A., Jiang, Q., McMahon, M., McWalter, G. K., McLellan, L. I., *et al*. (2002). Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase *Gsta1*, *Gsta2*, *Gstm1*, *Gstm2*, *Gstm3* and *Gstm4* genes in the livers of male and female mice. *Biochem. J.* **365**, 405–416. doi:10.1042/BJ20020320

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J. F., Rizos, D., and Lonergan, P. (2009). Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium. *Reproduction*, in press. doi:10.1530/REP-09-0152

Cran, D. G., Johnson, L. A., Miller, N. G., Cochrane, D., and Polge, C. (1993). Production of bovine calves following separation of X- and Y-chromosome bearing sperm and *in vitro* fertilisation. *Vet. Rec.* **132**, 40–41.

El-Sayed, A., Hoelker, M., Rings, F., Salilew, D., Jennen, D., Tholen, E., Sirard, M. A., Schellander, K., and Tesfaye, D. (2006). Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol. Genomics* **28**, 84–96. doi:10.1152/PHYSIOLGENOMICS.00111.2006

Fernández-Gonzalez, R., Moreira, P. N., Pérez-Crespo, M., Sánchez-Martín, M., Ramirez, M. A., *et al*. (2008). Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol. Reprod.* **78**, 761–772. doi:10.1095/BIOLREPROD.107.065623

Frijters, A. C., Mullaart, E., Roelofs, R. M., van Hoorne, R. P., Moreno, J. F., Moreno, O., and Merton, J. S. (2009). What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? *Theriogenology* **71**, 64–67. doi:10.1016/J.THERIOGENOLOGY.2008.09.025

Garner, D. L. (2006). Flow cytometric sexing of mammalian sperm. *Theriogenology* **65**, 943–957. doi:10.1016/J.THERIOGENOLOGY.2005.09.009

Garrett, J. E., Geisert, R. D., Zavy, M. T., and Morgan, G. L. (1988). Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J. Reprod. Fertil.* **84**, 437–446. doi:10.1530/JRF.0.0840437

Grant, V. J., Irwin, R. J., Standley, N. T., Shelling, A. N., and Chamley, L. W. (2008). Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biol. Reprod.* **78**, 812–815. doi:10.1095/BIOLREPROD.107.066050

Gutiérrez-Adán, A., Behboodi, E., Andersen, G. B., Medrano, J. F., and Murray, J. D. (1996). Relationship between stage of development and sex of bovine IVM-IVF embryos cultured *in vitro* versus in the sheep oviduct. *Theriogenology* **46**, 515–525. doi:10.1016/0093-691X(96)00173-2

Gutiérrez-Adán, A., Oter, M., Martínez-Madrid, B., Pintado, B., and De La Fuente, J. (2000). Differential expression of two genes located on the X chromosome between male and female *in vitro*-produced bovine embryos at the blastocyst stage. *Mol. Reprod. Dev.* **55**, 146–151. doi:10.1002/(SICI)1098-2795(200002)55:2<146::AID-MRD3>3.0.CO;2-F

Gutiérrez-Adán, A., Perez-Crespo, M., Fernandez-Gonzalez, R., Ramirez, M. A., Moreira, P., Pintado, B., Lonergan, P., and Rizos, D. (2006). Developmental consequences of sexual dimorphism during pre-implantation embryonic development. *Reprod. Domest. Anim.* **41**(Suppl. 2), 54–62. doi:10.1111/J.1439-0531.2006.00769.X

Hayakawa, H., Hirai, T., Takimoto, A., Ideta, A., and Aoyagi, Y. (2009). Superovulation and embryo transfer in Holstein cattle using sexed sperm. *Theriogenology* **71**, 68–73. doi:10.1016/J.THERIOGENOLOGY.2008.09.016

Hollinshead, F. K., Gillan, L., O'Brien, J. K., Evans, G., and Maxwell, W. M. (2003). *In vitro* and *in vivo* assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. *Reprod. Fertil. Dev.* **15**, 351–359. doi:10.1071/RD03060

Johnson, L. A., Welch, G. R., and Rens, W. (1999). The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for *in vitro* fertilization and AI. *J. Anim. Sci.* **77**(Suppl. 2), 213–220.

Karabinus, D. S. (2009). Flow cytometric sorting of human sperm: MicroSort® clinical trial update. *Theriogenology* **71**, 74–79. doi:10.1016/J.THERIOGENOLOGY.2008.09.013

Klinc, P., and Rath, D. (2007). Reduction of oxidative stress in bovine spermatozoa during flow cytometric sorting. *Reprod. Domest. Anim.* **42**, 63–67. doi:10.1111/J.1439-0531.2006.00730.X

Kobayashi, S., Isotani, A., Mise, N., Yamamoto, M., Fujihara, Y., *et al*. (2006). Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, *Rhox5/Pem*, at preimplantation stages. *Curr. Biol.* **16**, 166–172. doi:10.1016/J.CUB.2005.11.071

Libbus, B. L., Perreault, S. D., Johnson, L. A., and Pinkel, D. (1987). Incidence of chromosome aberrations in mammalian sperm stained with Hoechst 33342 and UV-laser irradiated during flow sorting. *Mutat. Res.* **182**, 265–274.

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., and Boland, M. P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* **117**, 159–167. doi:10.1530/JRF.0.1170159

Lu, K. H., Cran, D. G., and Seidel, G. E., Jr (1999). *In vitro* fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology* **52**, 1393–1405. doi:10.1016/S0093-691X(99)00225-3

Luna, M., Duke, M., Copperman, A., Grunfeld, L., Sandler, B., and Barritt, J. (2007). Blastocyst embryo transfer is associated with a sex-ratio imbalance in favor of male offspring. *Fertil. Steril.* **87**, 519–523. doi:10.1016/J.FERTNSTERT.2006.06.058

Mann, G. E., Fray, M. D., and Lamming, G. E. (2006). Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet. J.* **171**, 500–503. doi:10.1016/J.TVJL.2004.12.005

Manna, L., Neglia, G., Marino, M., Gasparrini, B., Di Palo, R., and Zicarelli, L. (2003). Sex determination of buffalo embryos (*Bubalus bubalis*) by polymerase chain reaction. *Zygote* **11**, 17–22. doi:10.1017/S0967199403001035

Maxwell, W. M., Long, C. R., Johnson, L. A., Dobrinsky, J. R., and Welch, G. R. (1998). The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. *Reprod. Fertil. Dev.* **10**, 433–440. doi:10.1071/RD98102

Maxwell, W. M. C., Evans, G., Hollinshead, F. K., Bathgate, R., de Graaf, S. P., Eriksson, B. M., Gillan, L., Morton, K. M., and O'Brien, J. K. (2004). Integration of sperm sexing technology into

the ART toolbox. *Anim. Reprod. Sci.* **82–83**, 79–95. doi:10.1016/J.ANIREPROSCI.2004.04.013

McNutt, T. L., and Johnson, L. A. (1996). Flow cytometric sorting of sperm: influence on fertilization and embryo/fetal development in the rabbit. *Mol. Reprod. Dev.* **43**, 261–267. doi:10.1002/(SICI)1098-2795(199602)43:2<261::AID-MRD16>3.0.CO;2-6

Meyer, C., Schmid, R., Scriba, P. C., and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. *Eur. J. Biochem.* **239**, 726–731. doi:10.1111/J.1432-1033.1996.0726U.X

Mocé, E., Graham, J. K., and Schenk, J. L. (2006). Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. *Theriogenology* **66**, 929–936. doi:10.1016/J.THERIOGENOLOGY.2006.01.063

Morton, K. M., Herrmann, D., Sieg, B., Struckmann, C., Maxwell, W. M. C., Rath, D., Evans, G., Lucas-Hahn, G., Niemann, H., and Wrenzycki, C. (2007). Altered mRNA expression patterns in bovine blastocysts after fertilisation *in vitro* using flow-cytometrically sex-sorted sperm. *Mol. Reprod. Dev.* **74**, 931–940. doi:10.1002/MRD.20573

Nedambale, T. L., Dinnyes, A., Yang, X., and Tian, X. C. (2004). Bovine blastocyst development *in vitro*: timing, sex, and viability following vitrification. *Biol. Reprod.* **71**, 1671–1676. doi:10.1095/BIOLREPROD.104.027987

Palma, G. A., Olivier, N. S., Neumuller, C., and Sinowatz, F. (2008). Effects of sex-sorted spermatozoa on the efficiency of *in vitro* fertilization and ultrastructure of *in vitro* produced bovine blastocysts. *Anat. Histol. Embryol.* **37**, 67–73.

Peluso, J. J., Pappalardo, A., Losel, R., and Wehling, M. (2006). Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. *Endocrinology* **147**, 3133–3140. doi:10.1210/EN.2006-0114

Peng, D. F., Razvi, M., Chen, H., Washington, K., Roessner, A., Schneider-Stock, R., and El-Rifai, W. (2009). DNA hypermethylation regulates the expression of members of the Mu-class glutathione S-transferases and glutathione peroxidases in Barrett's adenocarcinoma. *Gut* **58**, 5–15. doi:10.1136/GUT.2007.146290

Rath, D., Moench-Tegeder, G., Taylor, U., and Johnson, L. A. (2009). Improved quality of sex-sorted sperm: a prerequisite for wider commercial application. *Theriogenology* **71**, 22–29. doi:10.1016/J.THERIOGENOLOGY.2008.09.027

Reszka, E., Wasowicz, W., and Gromadzinska, J. (2007). Antioxidant defense markers modulated by glutathione S-transferase genetic polymorphism: results of lung cancer case-control study. *Genes Nutr.* **2**, 287–294. doi:10.1007/S12263-007-0057-Y

Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248. doi:10.1002/MRD.1153

Rizos, D., Bermejo-Alvarez, P., Gutierrez-Adan, A., and Lonergan, P. (2008). Effect of duration of oocyte maturation on the kinetics of cleavage, embryo yield and sex ratio in cattle. *Reprod. Fertil. Dev.* **20**, 734–740. doi:10.1071/RD08083

Schenk, J. L., and Seidel, G. E., Jr (2007). Pregnancy rates in cattle with cryopreserved sexed spermatozoa: effects of laser intensity, staining conditions and catalase. *Soc. Reprod. Fertil. Suppl.* **64**, 165–177.

Schenk, J. L., Suh, T. K., Cran, D. G., and Seidel, G. E., Jr (1999). Cryopreservation of flow-sorted bovine spermatozoa. *Theriogenology* **52**, 1375–1391. doi:10.1016/S0093-691X(99)00224-1

Seidel, G. E., Jr (2009). Sperm sexing technology: the transition to commercial application. An introduction to the symposium 'Update on sexing mammalian sperm'. *Theriogenology* **71**, 1–3. doi:10.1016/J.THERIOGENOLOGY.2008.09.015

Strange, R. C., Lear, J. T., and Fryer, A. A. (1998). Polymorphism in glutathione S-transferase loci as a risk factor for common cancers. *Arch. Toxicol. Suppl.* **20**, 419–428.

Suh, T. K., Schenk, J. L., and Seidel, G. E., Jr (2005). High pressure flow cytometric sorting damages sperm. *Theriogenology* **64**, 1035–1048. doi:10.1016/J.THERIOGENOLOGY.2005.02.002

Tiffin, G. J., Rieger, D., Betteridge, K. J., Yadav, B. R., and King, W. A. (1991). Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. *J. Reprod. Fertil.* **93**, 125–132. doi:10.1530/JRF.0.0930125

Tubman, L. M., Brink, Z., Suh, T. K., and Seidel, G. E., Jr (2004). Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. *J. Anim. Sci.* **82**, 1029–1036.

Virro, M. R., Larson-Cook, K. L., and Evenson, D. P. (2004). Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil. Steril.* **81**, 1289–1295. doi:10.1016/J.FERTNSTERT.2003.09.063

Ward, F., Enright, B., Rizos, D., Boland, M., and Lonergan, P. (2002). Optimization of *in vitro* bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology* **57**, 2105–2117. doi:10.1016/S0093-691X(02)00696-9

Xu, J., Guo, Z., Su, L., Nedambale, T. L., Zhang, J., *et al*. (2006). Developmental potential of vitrified holstein cattle embryos fertilized *in vitro* with sex-sorted sperm. *J. Dairy Sci.* **89**, 2510–2518.

Xu, J., Chaubal, S. A., and Du, F. (2009). Optimizing IVF with sexed sperm in cattle. *Theriogenology* **71**, 39–47. doi:10.1016/J.THERIOGENOLOGY.2008.09.012

Xu, K. P., Yadav, B. R., King, W. A., and Betteridge, K. J. (1992). Sex-related differences in developmental rates of bovine embryos produced and cultured *in vitro*. *Mol. Reprod. Dev.* **31**, 249–252. doi:10.1002/MRD.1080310404

Yadav, B. R., King, W. A., and Betteridge, K. J. (1993). Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated *in vitro*. *Mol. Reprod. Dev.* **36**, 434–439. doi:10.1002/MRD.1080360405

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I., and Sinclair, K. D. (2001). Epigenetic change in *IGF2R* is associated with fetal overgrowth after sheep embryo culture. *Nat. Genet.* **27**, 153–154. doi:10.1038/84769

Zhang, L., Kanda, Y., Roberts, D. J., Ecker, J. L., Losel, R., Wehling, M., Peluso, J. J., and Pru, J. K. (2008). Expression of progesterone receptor membrane component 1 and its partner serpine 1 mRNA binding protein in uterine and placental tissues of the mouse and human. *Mol. Cell. Endocrinol.* **287**, 81–89. doi:10.1016/J.MCE.2008.02.012

Manuscript received 14 June 2009, accepted 8 August 2009

CHAPTER 4

EPIGENETIC DIFFERENCES BETWEEN MALE AND FEMALE BOVINE BLASTOCYSTS PRODUCED IN VITRO

Physiol Genomics 32: 264–272, 2008.
First published November 6, 2007; doi:10.1152/physiolgenomics.00234.2007.

Epigenetic differences between male and female bovine blastocysts produced in vitro

P. Bermejo-Álvarez,[1] D. Rizos,[1] D. Rath,[3] P. Lonergan,[2] and A. Gutierrez-Adan[1]

[1]*Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain;* [2]*School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland; and* [3]*Department of Biotechnology, Institute of Animal Breeding (FAL), Neustadt, Germany*

Submitted 8 October 2007; accepted in final form 2 November 2007

Bermejo-Álvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol Genomics* 32: 264–272, 2008. First published November 6, 2007; doi:10.1152/physiolgenomics.00234.2007.—Epigenetic differences between male and female bovine blastocysts provide a plausible link between physiological and gene transcription differences observed between male and female embryos. The aim of this study was to examine sex-related epigenetic differences in bovine blastocysts produced in vitro. Oocytes were matured in vitro and inseminated with frozen-thawed sex-sorted (X or Y) and unsorted (control) bull sperm. Zygotes were cultured to blastocyst stage and were analyzed for embryo sexing, mtDNA content, telomere lengths, methylation analysis, and quantification of mRNA transcripts of DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) HMT1 hnRNP methyltransferase-like 2 (Hmt1), and interleukin enhancer binding factor 3 (Ilf3). There was a difference ($P < 0.05$) in the mean mtDNA copy number between male (410,000 ± 23,000) and female (360,000 ± 21,000) blastocysts. Telomere length was shorter in male blastocysts ($P < 0.01$). The level of methylation in a sequence near a variable number of tandem repeats minisatellite region [variable number of tandem repeats (VNTR)] in males (39.8% ± 4.8) was higher than in females (23.7% ± 3.1) ($P < 0.05$); however, no differences were found in other regions analyzed. Moreover, transcription differences between sexes were observed for Dnmt3a, Dnmt3b, Hmt1, and Ilf3. These results provide evidence of epigenetic differences between male and female bovine in vitro produced embryos and suggest that before initiation of gonadal differentiation, epigenetic events may modulate the difference between speed of development, metabolism, and transcription observed during preimplantation development between male and female embryos.

mtDNA; methylation; mRNA transcription

IN EUTHERIAN MAMMALS, EVIDENCE has emerged that clearly demonstrates differences in growth rates and metabolism between male and female embryos that appear before sexual differentiation of the gonads and, therefore, could not be explained by sex-related hormonal differences (6, 36). In preimplantation bovine embryos, total glucose metabolism is twice as high in male embryos as female embryos, and the activity of the pentose phosphate pathway is four times greater in female than in male blastocysts (54). Similar metabolic differences were found in human embryos at this stage (46). Differential metabolism and growth rates may be attributable to the unbalanced expression of X-linked genes between the sexes during certain stages of early preimplantation development, where both X chromosomes may be active (32, 40). We have shown that mRNA relative abundance of three X-linked genes are expressed at higher levels in female bovine embryos than in male embryos at the early blastocyst stage: two are important components of energetic metabolism, also involved in controlling the amount of oxygen radicals [glucose 6-phosphate dehydrogenase (G6PD) and hypoxanthine phosphoribosyl transferase (HPRT)] and the third, X-linked inhibitor of apoptosis protein (XIAP), is a mammalian protein that controls apoptosis through modulation of caspase activation and activity (19, 24). This differential expression has also been confirmed in other species (53, 58). The development of genomic procedures such as transgenesis and microarray analysis have allowed the discovery of nearly 600 differentially expressed genes between male and female mouse blastocysts (29). These results confirm differences previously reported in cattle (17).

Evidence from several species indicates that embryos produced in vitro that reach the blastocyst stage earliest are more likely to be males than females; examples include the mouse (55), cow (1, 17), human (44), pig (8), and sheep (4). Furthermore, the faster-developing blastocysts in in vitro culture systems are generally considered more viable and better able to survive cryopreservation or embryo transfer than those that develop more slowly (38). However, under suboptimal conditions, female embryos are more resistant than male embryos (43). This suggests that some early differences between male and female embryos are manifested under certain environmental conditions (18), and these early differences may be related to the control of the secondary sex ratio in mammals (i.e., differences in sex ratio observed at birth) (23). The mechanism(s) responsible for the observed phenotypic differences between male and female embryos in rate of development to the blastocyst stage is not clearly understood. Mitochondria, which play a central role in the provision of energy to embryo, may play a role in the differences in development between the male and female embryos (37), because enhanced rates of cell proliferation in the developing male embryo may be expected to require increased levels of cellular energy. Moreover it has been reported that mitochondria distribution at preimplantation stage may be an epigenetic factor developmentally relevant with respect to embryo competence (56). In addition, telomere elongation during embryogenesis is restricted to the preimplantation morula-blastocyst transition (49), and it is possible that the differences between sexes at early mammalian embryos have a telomerase-dependent genetic program that elongates telomeres to a defined length (49). Telomere length is also related with the epigenetic status of mammalian cells. It has been reported that telomere length regulates the epigenetic

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

Address for reprint requests and other correspondence: A. Gutiérrez-Adán, Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain (e-mail: agutierr@inia.es).

status (histone modifications and DNA methylation) of mammalian telomeres and subtelomeres (3). Also, during the preimplantation period, there is a relationship between genetic and epigenetic reprogramming (13); for this reason one could expect that the changes observed in gene expression between male and female embryo may be a cause or consequence of changes in epigenetic events. These epigenetic events may have a long term sex-linked effect in the adult animal (12) or may be hereditary and lead to sex-specific transgenerational responses (42).

Because in vitro culture may be responsible for or, at the very least, exacerbate the sex differences observed in embryos, bovine in vitro culture represents an excellent model to analyze genetic and epigenetic differences between male and female embryos because the oocyte takes >7 days to develop to blastocyst stage; this long period should help to amplify these differences (20) The aim of this study was to examine sex-related differences in mtDNA content, telomere length, methylation of different regions of the genome, and mRNA transcription of genes related with cytosine methylation and histone methylation in bovine blastocysts produced in vitro.

MATERIALS AND METHODS

Semen preparation. Semen was collected from a Holstein Frisian bull of proven fertility and diluted immediately with Sexcess extender (Masterrind, Verden, Germany) to a concentration of 1×10^8 sperm/ml. Spermatozoa were labeled with 15–25 μl of a 8.12 mM Hoechst 33342 solution [8.9 mM of 2-(-4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole in bi-distillated water] for 90 min at 34°C. Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (25). Labeled sperm samples were filtered through a 51 μm cell strainer grid (Falcon Becton Dickinson, Franklin Lakes, NJ) and then supplemented with 1 μl food dye solution FD&C#40 (Warner Jekinson, St. Louis, MO). Sorting was performed with a high-speed flow cytometer (MoFlo SX, DakoCytomation, Fort Collins, CO), equipped with an argon UV-Laser (Coherent Laser, Inova I 90C-6; Dieburg, Germany), set to 200 mW output. Samples were sorted at an average event rate of 25,000 cells/s, giving a sorting rate of 3,300 cells/s. Spermatozoa were collected into 10 ml conical plastic tubes (Greiner, Nürtingen, Germany) prefilled with 500 μl TEST-yolk extender (25). Immediately after collection of 8 million spermatozoa, the sorted cells were centrifuged at 840 *g* for 20 min. The supernatant was discharged, and the pellet was resuspended with a TRIS-based cooling extender and cooled to 4°C within 2 h. Then, the final sperm concentration was set 20.6×10^6 sperm/ml with a TRIS-based freezing extender (28), and 3.3×10^6 spermatozoa were filled into 0.25 ml plastic straws (Minitüb, Tiefenbach, Germany) (*segment 1* with 160 μl sperm and *segment 2* with 50 μl extender), sealed and frozen in liquid nitrogen. From each sorted frozen sample a purity analysis for the correct sex separation was performed using a flow cytometrical resort protocol and by curve fitting statistics [Gaus 7, (25)].

Blastocyst production. Immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows at slaughter. COCs were matured for 24 h in TCM-199 supplemented with 10% (vol/vol) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. For in vitro fertilization (IVF), matured COCs were inseminated with frozen-thawed, Percoll-separated, flow-cytometrically sex-sorted (X-sorted; $n = 1{,}057$ or Y-sorted; $n = 1{,}094$) and unsorted (control; $n = 157$) bull sperm at a concentration of 1×10^6 spermatozoa/ml. On each day of IVF a small number of oocytes were inseminated with unsorted semen as a control to ensure procedures in the laboratory were optimal, hence the lower numbers. Gametes were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At ~20 h postinsemination (hpi), presumptive zygotes were denuded and transferred to 25 μl culture droplets (1 embryo per μl) under mineral oil. Culture took place in synthetic oviduct fluid + 5% FCS. Plates were incubated for 7 days at 39°C under an atmosphere of 5% CO_2, 90% of N_2, and 5% O_2 with maximum humidity. *Day 7* blastocysts from both experimental groups were snap-frozen in groups of 10 for analysis of mRNA relative abundance, mtDNA, and methylation status. Five replicates were performed, and embryos from several replicates were used in the genetic and epigenetic analyses.

Embryo sexing by PCR. A preliminary study was performed for verification of the sorting procedure. All blastocysts used were produced in vitro as described above. *Day 7* blastocysts from both groups (X-sorted; $n = 47$, Y-sorted; $n = 61$) were first washed in PBS and then transferred into 5 mg/ml Pronase (Sigma P5147, Madrid, Spain) in PBS medium for 1 min to remove the zona pellucida and any attached spermatozoa. They were then washed three or four times in PBS and individually snap-frozen in liquid nitrogen in Eppendorf tubes and stored at −80°C until analysis. Samples were thawed at room temperature and centrifuged at 8,000 *g* for 1 min prior to being mixed with PCR reagents. Two sets of PCR primers were used to determine embryo sex: Y-chromosome-specific primers (BRY4a) and bovine-specific satellite sequence primers (Sat1) (33). Because of the number of repetitions of these sequences, this is one of the best systems to sex bovine embryos in a single PCR. The amplification reactions were conducted in a total volume of 25 μl containing 1× PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 1 unit of Taq DNA polymerase, 0.1 mM of the Sat1 primer, and 0.3 mM of the Bry4a primer. PCR was programmed for 35 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 20 s; in the first cycle denaturation was at 95°C for 3 min, and after the 35 cycles the reaction mixtures were kept at 72°C for 5 min. PCR product were analyzed on 2% agarose gel and ethidium bromide staining. The gel was visualized under ultraviolet illumination for the positive 300 bp band of Bry4a 1a and 216 bp of the satellite sequence. Samples that exhibited both bands were assigned as males, while the samples exhibiting only a satellite sequence band were assigned as female (Fig. 1).

Quantification of mtDNA. To analyze mtDNA, 60 individual *day 7* blastocysts and six groups of five blastocysts of each sex from three different experimental replicates were used. DNA was extracted form each sample and used directly for PCR analysis as described by Shitara et al. (50) and divided in three aliquots; each aliquot was used directly for sexing, mtDNA quantification (35), and measurement of telomere length by PCR. For mtDNA quantification, we used primers located in the COX1 gene (Table 1) that amplify a product of 190 bp (there is only one copy of the gene in the mitochondria genome). Quantification was performed by a real-time polymerase chain reaction (qRT-PCR) method. Briefly, a Rotorgene 2000 Real Time CyclerTM (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eurogene, OR) as a double-stranded DNA-specific fluorescent dye were used to determine the mtDNA copy number. The PCR reaction mixture (25 μl) contained: 1× PCR buffer, 3 mM $MgCl_2$, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 μM of each dNTPs, and 0.2 μM of each primer. In addition, the double-stranded DNA dye, SYBR Green I, (1:3,000 of 10,000× stock solution) was included in each reaction. The PCR protocol included an initial step of 94°C (2 min), followed by 40 cycles of 94°C (15 s), 56°C (30 s) and 72°C (10 s). Fluorescent data were acquired at 83°C after the elongation step. The melting protocol consisted of a hold temperature at 40°C for 60 s and then heating from 50 to 94°C, holding at each temperature for 5 s while monitoring fluorescence. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. The external standard was a COX1 PCR standard product that was purified and quantified by spectrophotometry, assuming that 1 ng of a 200 bp product contained 4.5×10^9 molecules of double-stranded DNA. Several serial dilutions were then

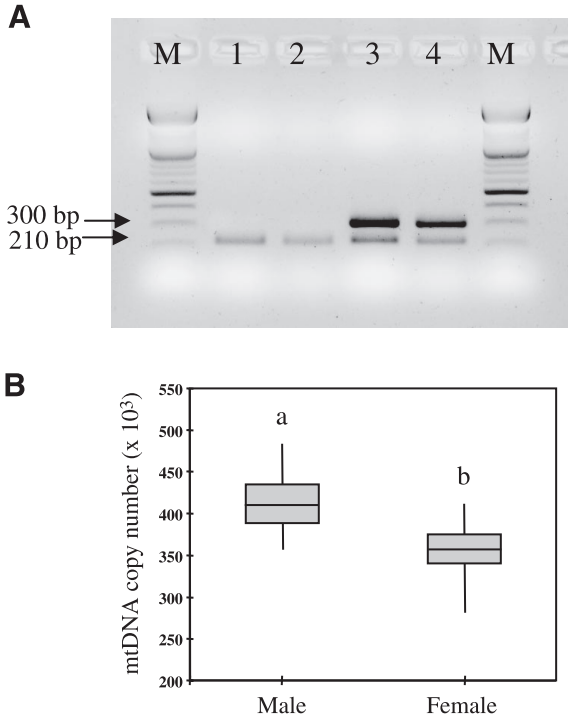


Fig. 1. *A*: representative results of the PCR sexing of embryos after agarose gel electrophoretic separation. A single PCR using both male-specific (Bry4a) and bovine Satellite (Sat) primers was carried out. *Lanes 1* and *2*: female embryos with a 216 bp specific product of Sat. *Lanes 3* and *4*: male embryos, a 300 bp Y chromosome-specific product (Bry4a) appeared in the *top* lane. M: 100 bp DNA ladder. *B*: comparison of mtDNA content between male and female bovine blastocysts. For each box, the central bar represents the mean; *top* and *bottom* boundaries of boxes represent ±1 SD, and vertical lines extend to the maximal and minimal values for each sex. Male in vitro produced blastocyst have more mtDNA than female (ANOVA analysis, $P < 0.05$).

made to assess the concentration of a known number of templates, and these were then used as external standards. For each replicate, a standard curve was generated using five 10-fold serial-dilutions (100–1,000,000 copies) of the external standard. This curve allowed the determination of the starting copy number of mtDNA in each sample. All samples were tested three times.

Quantification of telomere length. Average telomere length was measured from 60 individual bovine *day 7* blastocysts DNA by a real-time quantitative PCR method previously described (7). DNA was extracted form each blastocysts as described by Shitara et al. (50) and divided in three aliquots; each aliquot was used directly for sexing, mtDNA quantification, and measurement of telomeres length by PCR analysis. Quantification of telomere length by real-time PCR was performed according to the relative standard curve protocol, doing a minimum of three repetitions for each sample. The assay measures an average telomere length ratio (ATLR) by quantifying telomeric DNA with specially designed primer sequences and dividing that amount by the quantity of a single-copy gene (H2a.z). To serve as a reference for standard curve calculation, an individual sample of bovine somatic DNA was serially diluted over a 25-fold range for the telomere PCR and over 16-fold range for the H2a.z. The relative amount of the telomere PCR product was divided by the relative amount of the H2a.z, and the ratio of telomere/H2a.z was calculated. The average of these ratios was reported as the ATLR. The primers used for RT-PCR are listed in Table 1.

Bisulphite treatment, PCR, and restriction analysis of PCR products. To analyze methylation status, three groups of 10 embryos of each sex from three different experimental replicates were used. Embryos were washed in PBS, placed in 1.5 ml Eppendorf tubes, snap-frozen, and stored at −80°C until they were analyzed. As a control, the DNA of ~1 mm^3 of ovarian stroma was extracted with phenol-chloroform and diluted. The DNA was treated with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Bisulphite-modified DNA was used to amplify each sequence. The methylated status of a part of the satellite I region, a part of the satellite II region, a part of the 18S rRNA sequence, a part of the Alu-like short interspersed nuclear element (SINE) art2, and sequence near a VNTR region were amplified using nested or hemi-nested PCR (26, 27). The first PCR consisted of one cycle of 94°C for 2 min, 45°C for 30 s and 72°C for 20 s, followed by 4 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 20 s, 35 cycles of 94°C for 15 s, 54°C for 30 s, and 72°C for 20 s, and a final step of 72°C for 8 min. The nested PCR was carried out using 1 μl of the product and consisted of a first step of 94°C for 2 min, 35 cycles of 94°C 20 s, 54°C 30 s, and 72°C 30 s, and a final step of 72°C for 5 min. Primers are indicated in Table 2. Cytokeratin gene promoter sequence was amplified using a nested PCR consisting of one cycle of 94°C for 3 min, 56°C for 40 s, and 72°C for 20 s, followed by 4 cycles of 93°C for 1 min, 56°C for 30 s, and 72°C for 20 s, 25 cycles of 94°C for 15 s, 57°C for 30 s, and 72 °C for 20 s, and a final step of 72°C for 8 min. From the PCR products, 15 μl were digested with 1 UI of *Aci*I restriction enzyme (New England Biolabs, Hitchin, Herts, UK) overnight at 37 °C for the promoter sequence of cytokeratin gene, satellite I,

Table 1. *Details of primers used for qRT-PCR*

Gene	Primer Sequence (5′-3′)	Fragment Size, bp	Annealing Temperature, °C	Gene Bank Accession No.
Histone H2a.z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	208	59	NM_174809
COX1	AAATAATATAAGCTTCTGACTCC TCCTAAAATTGAGGAAACTCCT	190	59	AY676873
Dnmt1	CGCATGGGCTACCAGTGCACCTT GGGCTCCCCGTTGTATGAAATCT	311	57	BC114063
Dnmt3a	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	57	AY271299
Dnmt3b	GCACGAGGGCAACATCAAATACG CTCCAGGACCTTCCCAGCAGC	532	57	NM_181813
Hmt1	CCCAAGCAGCTGGTCACCAATG TCCAGGTCACGGTTGTTCTTGGC	320	59	BC109796
Ilf3	TATGGAGGCAACTCAGCGACTGC AGCCTTTCTGCTTGCCCTGTGG	301	59	XM_592855
Telomere	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	79	56	NT_039202

Dnmt, DNA methyltransferase; Hmt1, hnRNP methyltransferase-like 2; Ilf, interleukin enhancer binding factor.

Table 2. *Primers used in PCR for amplification of various target sequences*

DNA Region	Primer Sequence (5′-3′)	Primary or Nested PCR
Satellite I	AATACCTCTAATTTCAAACT	1st
	TTTGTGAATGTAGTTAATA	1st
	TTTCAAACTCCAATCACAAA	2nd
	TTAATATGAAGGGGTAGTTTTT	2nd
Satellite II	CAACCCATAATCAATAAACTC	1st
	GTTGAGGTAGTAGTTAGGTA	1st
	ATAAATCCACTTTAAATCCTTC	2nd
	GGGATATTTAGTTAAGAGTAT	2nd
Art2	TTAAATTCAATTCAATCACTCAATCA	1st
	TTTATGTGAAGAGTTGATTTATTGGA	1st
	CCAAACCTCCCTATCCATCTC	2nd
	TTTATGTGAAGAGTTGATTTATTGGA	2nd
VNTR	CAACTCAAAATAACCCACTATC	1st
	GTGTTTATGGTGATAGTAGTG ATGA	1st
	CACTATCCTATCAAACTTATC	2nd
	TTTATGTGAAGAGTTGATTTATTGGA	2nd
Cytokeratin	CCTCTTTCTACCAAACAAACCAA	1st
	GTGGATGGTAAGTTATTTAAAAGGAG	1st
	ACAAACCAAAAACTAATAATACCTCA	2nd
	TAAAAGGAGAGTGGATTGGGAG	2nd
18S rRNA	GGGATATTTAGTTAAGAGTAT	1st and 2nd
	AACTCTTTCRAAACCCTATAAT	1st
	ATAAATCCACTTTAAATCCTTC	2nd

VNTR, variable number of tandem repeats.

18S rRNA and VNTR; with 1 UI of *Mvn*I restriction enzyme (New England Biolabs) overnight at 37 °C for satellite II; or with 1 UI of TaqI restriction enzyme (New England Biolabs) overnight at 55 °C for art2. As a control, another 15 μl of the PCR products were mixed with restriction enzyme buffer without enzyme and exposed at 37°C or 55°C overnight. Digested and undigested samples were resolved in 2% agarose gel stained with ethidium bromide. Methylation status of the sequences analyzed was obtained by comparison of the relative DNA amount between digested and undigested samples. Once the bands were separated by electrophoresis, a picture of the gel was taken, avoiding pixel saturation, to obtain the relative DNA amount of each band by pixel density using ScionImage software. To determine pixel density, the image was inverted, then the selection tool was used to select the bigger band, and the same selected area was used for every band. The mean of pixel density in each area was obtained via histogram, and pixel density of each band was calculated, removing background pixel density. Linear regression of band mass versus pixel density was assessed by plotting a standard curve with increasing amounts of the PCR products of each gene. Results were finally obtained by comparison of pixel density between the undigested band of the digested DNA and the unique band of the undigested PCR product.

RNA extraction, reverse transcription, and quantification of mRNA transcript abundance. Poly(A) RNA was prepared from five groups of pools of 10 blastocysts from each experimental group (males or females) following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway). Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Promega, Madrid, Spain) using poly(T) anchor primer and AMV reverse transcriptase enzyme in a volume of 20 μl to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 5 units of Superscript RT enzyme. They were then incubated at room temperature for 10 min and then at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme. We used 4 μl of the cDNA sample in the RT-PCR for the detection of each transcript.

The quantification of all mRNA transcripts was carried out by real-time qRT-PCR. Five replicate PCR experiments were conducted for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone H2a.z in every sample. PCR was performed by adding a 4 μl aliquot of each sample to the PCR mix containing the specific primers to amplify H2a.z, DNA methyltransferase (Dnmt) 1, Dnmt3a, Dnmt3b, hnRNP methyltransferase-like 2 (Hmt1), and interleukin enhancer binding factor 3 (Ilf3). Primer sequences, annealing temperature, and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, real time PCR was performed as described above. The comparative cycle threshold (CT) method was used to quantify expression levels (12). Quantification was normalized to the endogenous control, H2A. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the H2a.z CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis. Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. Cleavage and embryo development was analyzed using one-way repeated-measures ANOVA with arcsine transformation. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student-Newman-Keuls method) was used for the analysis of mtDNA, percentage of methylation, and differences in mRNA expression assayed by quantitative RT-PCR. The mean of the male and female telomere length were compared using an independent samples *t*-test.

RESULTS

In vitro embryo development and sex ratio of bovine embryos. Sexing was performed on zonafree embryos with a single PCR using the male-specific primer, Bry4a, and a satellite Sat1. The proportion of female and male blastocysts obtained with X- and Y-chromosome-bearing sperm in the preliminary study was 87.2 and 80.3%, respectively (Fig. 1*A*, Table 3). The proportion of zygotes cleaving at 48 hpi was not different between X- and Y-sorted groups (58.2 vs. 55.1%, respectively); however, both groups were significantly different from the unsorted group (86.0%. $P < 0.001$). Furthermore, the proportion of blastocysts formed on *day 7* and *8* followed the same trend; there was no significant difference between X- and Y-sorted groups (*day 7*: 10.1% vs. 13.5%, *day 8*: 13.8% vs. 18.3%, respectively) but significantly more blastocysts were produced in the unsorted group (*day 7*: 51.6%, *day 8*: 55.4%, $P < 0.001$; Table 4).

mtDNA copy number and telomere length in male and female blastocysts. For mtDNA quantification we used single and pooled blastocysts. A lower intersample variation was

Table 3. *Effect of using sex-sorted bovine sperm in IVF on the production of male or female blastocysts in vitro on day 7*

	Blastocysts Sexed, *n*	Females, *n* (%)	Males, *n* (%)
X-sorted	47	41 (87.2)	6 (12.8)
Y-sorted	61	12 (19.7)	49 (80.3)

IVF, in vitro fertilization.

Table 4. *Effect of using sex-sorted bovine semen in IVF on the cleavage rate and blastocyst yield in vitro*

			Blastocysts on *Day 7*, *n*		Blastocysts on *Day 8*, *n*	
	COCs, *n*	Cleaved, *n* (%)	Total* (%)	Cleaved# (%)	Total (%)	Cleaved (%)
Control†	157	135[a] (86.0)	81/157[a] (51.6)	81/135[a] (60.0)	87/157[a] (55.4)	87/135[a] (64.4)
X-sorted	1,057	615[b] (58.2)	107/1,057[b] (10.1)	107/615[b] (17.4)	146/1,057[b] (13.8)	146/615[b] (23.7)
Y-sorted	1,094	603[b] (55.1)	148/1,094[b] (13.5)	148/603[b] (24.5)	200/1,094[b] (18.3)	200/603[b] (33.2)

[a,b]Values in the same column differ significantly ($P < 0.05$). Data from 5 experimental replicates. †On each day of IVF a small number of oocytes were inseminated with unsorted semen as a control to ensure procedures in the laboratory were optimal, hence the lower numbers. *Number of blastocysts from the total number of cumulus oocytes complexes (COCs); #number of blastocysts from the cleaved oocytes.

found when we used pooled embryos; however, in both cases the differences between sexes were significant. The mean mtDNA copy number from the pool analysis of male and female bovine blastocysts is shown in Fig. 1*B*. There was a difference between genders ($P < 0.05$); mtDNA content average in male blastocysts was 410,000 ± 23,000 and in females was 360,000 ± 21,000. When individual male and female blastocysts were analyzed the mtDNA content average in male blastocysts was 423,000 ± 33,000 and in females was 373,000 ± 27,000.

For telomere length quantification we used single Day 7 blastocysts. To evaluate the real-time PCR method, DNA from the tail of two mouse species, *Mus musculus* and *M. spretus*, was used (*M. musculus* animals have long telomeres with repeats of >20 kb, and *M. spretus* mice have short telomeres, similar to those in bovine, with 5–10 kb repeats). The mean ATLRs for the two species were compared and found to be statistically different. The mean ATLRs and the average standard deviation for the ATLRs for the two groups were similar to those reported by Callicott and Womack (7). No PCR products were noted when the genomic DNA template was omitted or when *Escherichia coli* DNA was substituted in the reaction (data non shown). Telomere length was shorter in male bovine blastocysts than in female blastocysts ($P < 0.01$, Fig. 2).

Differential methylation between male and female blastocysts. To examine the methylation status of male or female bovine blastocysts, purified genomic DNA from male and female embryos was treated with bisulphite, which causes deamination of unmethylated cytosines to uridine, thereby allowing discrimination between unmethylated and methylated cytosine residues through restriction enzyme analysis. The bisulphite-treated DNAs were subjected to PCR, and products were digested by restriction enzyme. We examined the methylation status of six genomic regions (that cover both euchromatic and heterochromatic DNA regions) in male and female bovine blastocysts produced in vitro (these regions have been previously used to analyzed differences in methylation between bovine blastocysts produced in vivo, in vitro, or by nuclear transfer) (26, 27): a region of the promoter of bovine epidermal cytokeratin gene, a sequence of the higher repeat satellite I region (*Sat*I), a sequence of the higher repeat satellite II region (*Sat*II), a sequence near a VNTR, a sequence near the euchromatic repeated sequence 18S rRNA, and a part of the euchromatic repeat sequence Art2. A 153 bp unique genomic sequence near a VNTR region which three *Aci*I recognition sites and eight CpG sequences was digested by *Aci*I enzyme, with recognizes only the unconverted (methylated) sequences. The sequence near VNTR region is highly methylated in sperm and become extensively demethylated at the morula and blastocyst stages (27). The level of methylation in males (39.8 ± 4.8%) was significantly higher ($P < 0.01$) than in females (23.7 ± 3.1%) (Fig. 3*A*). In contrast, the rest of the analyzed sequences were hypomethylated in both sexes, but Art2, which was partially methylated and did not show any difference between sexes (males 22.6 ± 7.7%, females 19.6 ± 4.5%).

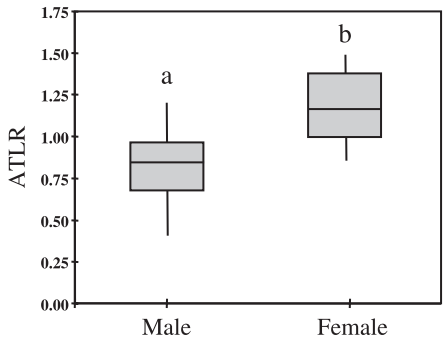


Fig. 2. Comparison of the average telomere length ratios (ATLR, means ± SE) between male and female bovine blastocysts, showing that the female group has longer telomeres. Means were compared by an independent sample *t*-test and were found to be significantly different ($P < 0.01$).

Relative abundance of selected gene transcripts. In a preliminary experiment to select a reference housekeeping gene for data normalization we analyzed mRNA abundance of β-actin (47), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (48), ribosomal protein L7 (Rpl7) (UniGene Bt.67188), and histone H2a.z (H2a.z) (21) between male and female bovine blastocysts. We found differences between male and female blastocysts for Rpl7, but we did not observe differences for the other housekeeping genes. For that reason, quantification was normalized to the endogenous control H2a.z.

There was no difference in the relative abundance of mRNA for Dnmt1 between male and female blastocysts. However, Hmt1 ($P < 0.01$), Ilf3 ($P < 0.01$), Dnmt3a ($P < 0.001$), and Dnmt3b ($P < 0.001$) were significantly upregulated in male blastocysts (Fig. 3*B*).

DISCUSSION

Bovine embryos derived from sex-sorted sperm have similar morphology and timing of development than those fertilized with unsorted sperm. However, at least in this study, sex-sorted bovine sperm have a lower fertility and lead to reduced embryo development compared with unsorted sperm when used in vitro. This is generally attributed to the deleterious effect of the sex-sorting procedure on the capacitation status and lifespan of sex-sorted sperm (34).

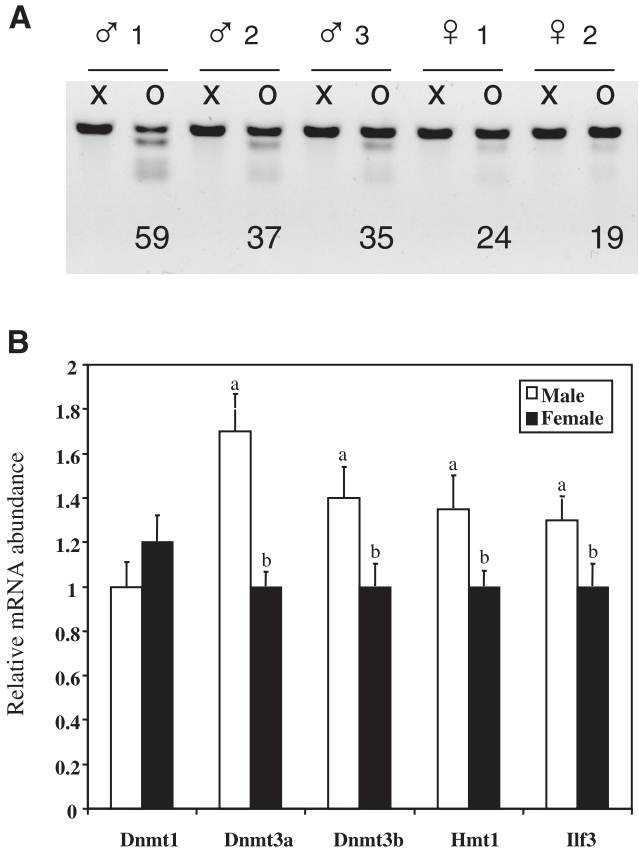


Fig. 3. *A*: representative result of differential methylation between male and female bovine blastocysts of a 179 bp PCR of a unique genomic sequence near a VNTR region digested with *Aci*I restriction enzyme (X, intact, undigested PCR products; O, enzyme-treated PCR products). Numbers are percentage digestion calculated from undigested band intensity of digested samples relative to that of the undigested sample. *B*: relative mRNA expression of several candidate genes related with cytosine methylation (Dnmt1, Dnmt3a, and Dnmt3b) or histone methylation (Hmt1 and Ilf3) in bovine male and female blastocysts produced in vitro. ANOVA analysis, $P < 0.01$. VNTR, variable number of tandem repeats; Dnmt, DNA methyltransferase; Hmt1, hnRNP methyltransferase-like 2; Ilf3, interleukin enhancer binding factor 3.

Quantitative variation in mtDNA has been associated with gamete quality and reproductive success. It has been reported that mitochondria and mtDNA genotype affect the developmental capacity of bovine oocytes in vitro (51) The mean copy number of mtDNA per blastocyst reported here is consistent with previous studies (35). It has been reported that the mtDNA copy number increases at the blastocyst stage in bovine embryos (35). The fact that male embryos have more copies of mtDNA indicates that this increase is faster in male than in female bovine embryos, or conversely, that degradation of mitochondria is higher in female than in male embryos during early development. It has been hypothesized that a divergence in energy metabolism is at the root of the differences between the sexes in mammals (37), and since metabolically active cells tend to contain more mitochondria than less active ones, there should be a difference in the number and/or activity of mitochondria in developing male and female mammals.

It has been reported in humans that telomeres on early male embryo Xqs are ~1,100 bp shorter than on female Xqs (45). In mice and rats it has been reported that telomere lengths are shorter in adult and new born males than in females (9, 10). It has been also been reported that early mammalian embryos have a telomerase-dependent genetic program that elongates telomeres to a defined length (49). Analysis revealed no significant increase in telomere length between *day 8* and *13.5* of mouse embryogenesis compared with length at the morula-blastocyst transition, indicating that telomere elongation during embryogenesis is restricted to the preimplantation morula-blastocyst transition (49). Because we have identified differences at the blastocyst stage between male and female embryos, it is possible that the differences between sexes at birth are consequences of the differences generated during the preimplantation period. We do not know the origin of these differences between sexes, but recently a locus with a major effect on telomere length on the distal X chromosome has been identified (7). One possibility is that this locus behaves like other described X-linked genes that are expressed at higher levels in female bovine embryos than in male embryos at the early blastocyst stage (19, 24). In addition, we have found that the expression of Dnmt3a and Dnmt3b is higher in male than in female bovine embryos, in agreement with reports that these methyltransferases are negative regulators of telomere length (16). Moreover, mouse embryonic stem cells genetically deficient for Dnmt3a and Dnmt3b have dramatically elongated telomeres compared with wild-type controls (16). Our results are in agreement with the link between epigenetic status and telomere-length regulation (5).

Also, we found that some epigenetic modifications take place differentially between male and female embryos. We did not observe differences in those sequences that were hypomethylated: the surrounding genomic heterochromatic repeats regions to *Sat*I and *Sat*II, a region of the euchromatic repeated sequence 18S rRNA and in a region of the promoter of cytokeratin gene, nor in a part of the euchromatic SINE element art2, that was partially methylated (males 22.6 ± 7.7%, females 19.6 ± 4.5%). These findings are similar to those reported by Kang et al. (26), who found considerably hypomethylated states in *Sat*I, 18S rRNA and the promoter of the cytokeratin gene and some degree of methylation (26%) in art2 in IVF blastocysts, except for *Sat*II, which was reported to be methylated to some degree (27.8%) (26). This difference can be attributed to the different DNA methylation analysis method used (cloning and sequencing versus restriction enzyme analysis), as similar differences between methods have been found in others sequences such as *Sat*I (26). However, we found differences in a unique sequence near a minisatellite repeat locus VNTR (AF012918) (26), indicating that the difference in methylation between male and female is not a genome-wide phenomenon and that there are sequence- or genomic region-specific differences in epigenetic modification between male and female bovine blastocysts. These findings present the possibility that other single-copy genes that are important for full-term development may also be differentially demethylated and may explain the differential expression of some autosomal genes (29, 30, 43). These repeat minisatellites (VNTR) are widespread within the genome and have been widely used as genetic markers, owing to the highly polymorphic nature of their tandem repeat number (22).

Sex-related differences in mRNA transcription of certain genes may implicate a new epigenetic process occurring in early embryos that precedes gonadal sex commitment. DNA

methylation is vital for preimplantation embryo development, necessary for imprinting, transposon silencing, X chromosome dosage compensation, and genome stability. Methylation of the cytosines is the predominant epigenetic modification of vertebrate genome. It is catalyzed by Dnmt enzymes; Dnmt1 is the major maintenance methyltransferase, and it ensures that newly synthesized DNA retains the methylation pattern of the template; Dnmt3a and Dnmt3b are de novo methyltransferases, setting up the methyl-CG landscape of the genome early in development. We have found similar expression of Dnmt1 between male and female bovine embryos, but lower expression of Dnmt3a and Dnmt3b in females. It has been reported that DNA methylation is lower in XX ES cell lines than in XY or XO lines and that this hypomethylation is associated with reduced levels of Dnmt3a and Dnmt3b (60). They speculate that the X chromosome encodes a modifier locus whose product represses de novo methyltransferases. Cells with two active X chromosomes will overexpress the modifier and therefore have reduced levels of the enzymes (60). Also, the influence of sex chromosome constitution on the genomic imprinting of germ cells has been reported (11). They found that there is a dose-dependent demethylating effect exerted by the X chromosome (one in XY, two in XX germ cells, with both X chromosomes active). In addition, the X-coded protein ATRX is known to be involved in chromatin modification, and is dosage sensitive (14). It has also been reported that during in vitro culture in preimplantation embryos there is higher expression of genes present on the X chromosome in female than in male embryos, indicating that two X chromosome are active (19, 29, 39, 58). This could suggest that female cells will overexpress the modifier and therefore have reduced levels of the enzyme. The differences in the expression of these methyltransferase genes between male and female embryos may be necessary to establish the differences observed in gene expression between genders that take place in early postimplantation embryos (29).

Methylation of specific residues within the NH_2-terminal histone tails plays a critical role in regulating eukaryotic gene expression. Ilf3 is a cell cycle-regulated protein that it is cyclically phosphorylated during mitosis (52) and regulates PRMT1 activity, the type I protein-arginine methyltransferase, that is a cofactor of nuclear receptor-activated gene expression, acting in the methylation of the histone 4 arginine 3 (2). Hmt1 is the bovine homolog to human and murine PRMT1. These protein arginine N-methyltransferases have been implicated in a variety of processes, including cell proliferation, signal transduction, and protein trafficking (41). Embryos homozygous mutant for this gene failed to develop beyond embryonic day (E) 6.5 (41), and the expression of the Prmt1 gene in wild-type mice was greatest along the midline of the neural plate and in the forming head fold from E7.5 to E8.5 and in the developing central nervous system from E8.5 to E13.5 (41). The early differences in expression that we have found could be related to the surprisingly widespread sexually dimorphic gene expression in mice, as manifested by the identification of thousands of differentially expressed genes between male and female mice (59), and differential gene expression between the developing brains of male and female mice at stage 10.5 days postcoitum, before any gonadal hormone influence.

The differences in growth, metabolism, and genetic and epigenetic programming during the preimplantation stages indicates that males and females may respond differently to environmental conditions and suggest that early perturbations may have a sex-specific effect, not only during preimplantation development, but also that may lead to some subsequent effects on postnatal development (20). Undesirable postnatal sex-associated phenotypic consequences can result from the alteration of long-term genetic or epigenetic reprogramming (15) as a consequence of embryo exposure to suboptimal in vitro culture conditions. Possibly related to this, Beckwith-Wiedemann syndrome associated with hypomethylation of the KvDMR1 (DMRs: differentially methylated regions) occurs at a relatively high frequency in monozygotic twins, and in almost all cases, the affected twins are female (31, 57). It will be important to examine this relationship further and also to determine whether female bias occurs in association with other diseases with an epigenetic component. Embryos of different sex may respond differently to epigenetic alterations. By analyzing these early sex differences we will be able to exert greater control on sex ratio manipulation of domestic animals, and it will help us to understand other aspects of early embryo development, X inactivation, and epigenetic and genetic processes related to early development that may have a long-term effect on the offspring.

GRANTS

This work was supported by Spanish Ministry of Science and Technology Grants, AGL2006-04799 to A. Gutiérrez-Adán and by AGL2006-05616 and AT2006-003 to D. Rizos. P. Bermejo-Álvarez was supported by an FPU grant from the Spanish Ministry of Education and Science.

REFERENCES

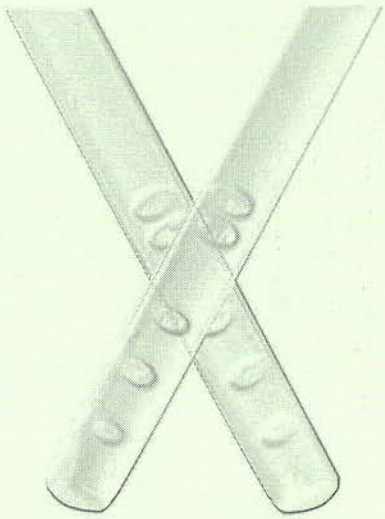
1. **Avery B, Jorgensen CB, Madison V, Greve T.** Morphological development and sex of bovine in vitro-fertilized embryos. *Mol Reprod Dev* 32: 265–270, 1992.
2. **Balint BL, Szanto A, Madi A, Bauer UM, Gabor P, Benko S, Puskas LG, Davies PJ, Nagy L.** Arginine methylation provides epigenetic transcription memory for retinoid-induced differentiation in myeloid cells. *Mol Cell Biol* 25: 5648–5663, 2005.
3. **Benetti R, Garcia-Cao M, Blasco MA.** Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nat Genet* 39: 243–250, 2007.
4. **Bernardi ML, Delouis C.** Sex-related differences in the developmental rate of in-vitro matured/in-vitro fertilized ovine embryos. *Hum Reprod* 11: 621–626, 1996.
5. **Blasco MA.** The epigenetic regulation of mammalian telomeres. *Nat Rev Genet* 8: 299–309, 2007.
6. **Burgoyne PS, Thornhill AR, Boudrean SK, Darling SM, Bishop CE, Evans EP.** The genetic basis of XX-XY differences present before gonadal sex differentiation in the mouse. *Philos Trans R Soc Lond B Biol Sci* 350: 253–260, 1995.
7. **Callicott RJ, Womack JE.** Real-time PCR assay for measurement of mouse telomeres. *Comp Med* 56: 17–22, 2006.
8. **Cassar G, King WA, King GJ.** Influence of sex on early growth of pig conceptuses. *J Reprod Fertil* 101: 317–320, 1994.
9. **Cherif H, Tarry JL, Ozanne SE, Hales CN.** Ageing and telomeres: a study into organ- and gender-specific telomere shortening. *Nucleic Acids Res* 31: 1576–1583, 2003.
10. **Coviello-McLaughlin GM, Prowse KR.** Telomere length regulation during postnatal development and ageing in Mus spretus. *Nucleic Acids Res* 25: 3051–3058, 1997.
11. **Durcova-Hills G, Hajkova P, Sullivan S, Barton S, Surani MA, McLaren A.** Influence of sex chromosome constitution on the genomic imprinting of germ cells. *Proc Natl Acad Sci USA* 103: 11184–11188, 2006.
12. **Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A.** Long-term effect of in vitro culture of mouse embryos

with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci USA* 101: 5880–5885, 2004.
13. **Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ.** The embryo and its future. *Biol Reprod* 71: 1046–1054, 2004.
14. **Gibbons RJ, McDowell TL, Raman S, O'Rourke DM, Garrick D, Ayyub H, Higgs DR.** Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat Genet* 24: 368–371, 2000.
15. **Gluckman PD, Hanson MA.** Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. *Pediatr Res* 56: 311–317, 2004.
16. **Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA.** DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat Cell Biol* 8: 416–424, 2006.
17. **Gutierrez-Adan A, Behboodi E, Andersen GB, Medrano JF, Murray JD.** Relationship between stage of development and sex of bovine IVM-IVF embryos cultured in vitro versus in the sheep oviduct. *Theriogenology* 46: 515–525, 1996.
18. **Gutierrez-Adan A, Granados J, Pintado B, De La Fuente J.** Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. *Reprod Fertil Dev* 13: 361–365, 2001.
19. **Gutierrez-Adan A, Oter M, Martinez-Madrid B, Pintado B, De La Fuente J.** Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. *Mol Reprod Dev* 55: 146–151, 2000.
20. **Gutierrez-Adan A, Perez-Crespo M, Fernandez-Gonzalez R, Ramirez M, Moreira P, Pintado B, Lonergan P, Rizos D.** Developmental consequences of sexual dimorphism during pre-implantation embryonic development. *Reprod Domest Anim* 41, *Suppl* 2: 54–62, 2006.
21. **Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP, Lonergan P.** Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol Reprod Dev* 68: 441–448, 2004.
22. **Haku T, Hibino T, Fukada H, Mishima Y, Yamashita I, Kato M.** DNA secondary structure forming at minisatellite repeat unit sequences. *Nucleic Acids Symp Ser (Oxf)*: 229–230, 2006.
23. **Ingemarsson I.** Gender aspects of preterm birth. *BJOG* 110, *Suppl* 20: 34–38, 2003.
24. **Jimenez A, Madrid-Bury N, Fernandez R, Perez-Garnelo S, Moreira P, Pintado B, de la Fuente J, Gutierrez-Adan A.** Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. *Mol Reprod Dev* 65: 180–187, 2003.
25. **Johnson LA, Welch GR, Rens W.** The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. *J Anim Sci 77, Suppl* 2: 213–220, 1999.
26. **Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM.** Aberrant methylation of donor genome in cloned bovine embryos. *Nat Genet* 28: 173–177, 2001.
27. **Kang YK, Park JS, Koo DB, Choi YH, Kim SU, Lee KK, Han YM.** Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. *EMBO J* 21: 1092–1100, 2002.
28. **Klinc P, Rath D.** Reduction of oxidative stress in bovine spermatozoa during flow cytometric sorting. *Reprod Domest Anim* 42: 63–67, 2007.
29. **Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, Nakanishi T, Ikawa M, Hamada H, Abe K, Okabe M.** Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. *Curr Biol* 16: 166–172, 2006.
30. **Larson MA, Kimura K, Kubisch HM, Roberts RM.** Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-tau. *Proc Natl Acad Sci USA* 98: 9677–9682, 2001.
31. **Lubinsky MS, Hall JG.** Genomic imprinting, monozygous twinning, and X inactivation. *Lancet* 337: 1288, 1991.
32. **Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N.** Reactivation of the paternal X chromosome in early mouse embryos. *Science* 303: 666–669, 2004.
33. **Manna L, Neglia G, Marino M, Gasparrini B, Di Palo R, Zicarelli L.** Sex determination of buffalo embryos (Bubalus bubalis) by polymerase chain reaction. *Zygote* 11: 17–22, 2003.
34. **Maxwell WM, Evans G, Hollinshead FK, Bathgate R, De Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK.** Integration of sperm sexing technology into the ART toolbox. *Anim Reprod Sci* 82–83: 79–95, 2004.
35. **May-Panloup P, Vignon X, Chretien MF, Heyman Y, Tamassia M, Malthiery Y, Reynier P.** Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. *Reprod Biol Endocrinol* 3: 65, 2005.
36. **Mittwoch U.** Blastocysts prepare for the race to be male. *Hum Reprod* 8: 1550–1555, 1993.
37. **Mittwoch U.** The elusive action of sex-determining genes: mitochondria to the rescue? *J Theor Biol* 228: 359–365, 2004.
38. **Nedambale TL, Dinnyes A, Yang X, Tian XC.** Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. *Biol Reprod* 71: 1671–1676, 2004.
39. **Nino-Soto MI, Basrur PK, King WA.** Impact of in vitro production techniques on the expression of X-linked genes in bovine (bos taurus) oocytes and pre-attachment embryos. *Mol Reprod Dev* 74: 144–153, 2007.
40. **Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E.** Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303: 644–649, 2004.
41. **Pawlak MR, Scherer CA, Chen J, Roshon MJ, Ruley HE.** Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. *Mol Cell Biol* 20: 4859–4869, 2000.
42. **Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, Golding J.** Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* 14: 159–166, 2006.
43. **Perez-Crespo M, Ramirez MA, Fernandez-Gonzalez R, Rizos D, Lonergan P, Pintado B, Gutierrez-Adan A.** Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Mol Reprod Dev* 72: 502–510, 2005.
44. **Pergament E, Fiddler M, Cho N, Johnson D, Holmgren WJ.** Sexual differentiation and preimplantation cell growth. *Hum Reprod* 9: 1730–1732, 1994.
45. **Perner S, Bruderlein S, Hasel C, Waibel I, Holdenried A, Ciloglu N, Chopurian H, Nielsen KV, Plesch A, Hogel J, Moller P.** Quantifying telomere lengths of human individual chromosome arms by centromere-calibrated fluorescence in situ hybridization and digital imaging. *Am J Pathol* 163: 1751–1756, 2003.
46. **Ray PF, Conaghan J, Winston RM, Handyside AH.** Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. *J Reprod Fertil* 104: 165–171, 1995.
47. **Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J, Gutierrez-Adan A.** Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol Reprod* 66: 589–595, 2002.
48. **Robert C, McGraw S, Massicotte L, Pravetoni M, Gandolfi F, Sirard MA.** Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol Reprod* 67: 1465–1472, 2002.
49. **Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL.** Telomere length is reset during early mammalian embryogenesis. *Proc Natl Acad Sci USA* 101: 8034–8038, 2004.
50. **Shitara H, Kaneda H, Sato A, Inoue K, Ogura A, Yonekawa H, Hayashi JI.** Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. *Genetics* 156: 1277–1284, 2000.
51. **Tamassia M, Nuttinck F, May-Panloup P, Reynier P, Heyman Y, Charpigny G, Stojkovic M, Hiendleder S, Renard JP, Chastant-Maillard S.** In vitro embryo production efficiency in cattle and its association with oocyte adenosine triphosphate content, quantity of mitochondrial DNA, and mitochondrial DNA haplogroup. *Biol Reprod* 71: 697–704, 2004.
52. **Tang J, Kao PN, Herschman HR.** Protein-arginine methyltransferase I, the predominant protein-arginine methyltransferase in cells, interacts with and is regulated by interleukin enhancer-binding factor 3. *J Biol Chem* 275: 19866–19876, 2000.
53. **Taylor DM, Handyside AH, Ray PF, Dibb NJ, Winston RM, Ao A.** Quantitative measurement of transcript levels throughout human preimplantation development: analysis of hypoxanthine phosphoribosyl transferase. *Mol Hum Reprod* 7: 147–154, 2001.

54. **Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA.** Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. *J Reprod Fertil* 93: 125–132, 1991.
55. **Valdivia RP, Kunieda T, Azuma S, Toyoda Y.** PCR sexing and developmental rate differences in preimplantation mouse embryos fertilized and cultured in vitro. *Mol Reprod Dev* 35: 121–126, 1993.
56. **Van Blerkom J, Davis P, Alexander S.** Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. *Hum Reprod* 15: 2621–2633, 2000.
57. **Weksberg R, Shuman C, Caluseriu O, Smith AC, Fei YL, Nishikawa J, Stockley TL, Best L, Chitayat D, Olney A, Ives E, Schneider A, Bestor TH, Li M, Sadowski P, Squire J.** Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. *Hum Mol Genet* 11: 1317–1325, 2002.
58. **Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H.** In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol Reprod* 66: 127–134, 2002.
59. **Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ.** Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Res* 16: 995–1004, 2006.
60. **Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R, Brockdorff N.** Global hypomethylation of the genome in XX embryonic stem cells. *Nat Genet* 37: 1274–1279, 2005.

CHAPTER 5

SEX DETERMINES THE EXPRESSION LEVEL OF ONE THIRD OF THE ACTIVELY EXPRESSED GENES IN BOVINE BLASTOCYSTS



Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts

P. Bermejo-Alvarez[a], D. Rizos[a], D. Rath[b], P. Lonergan[c], and A. Gutierrez-Adan[a,1]

[a]Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid 28040, Spain; [b]Institute of Farm Animal Genetics, Friedrich-Loeffer-Institut, 31535 Neustadt-Mariensee, Germany; and [c]School of Agriculture, Food Science, and Veterinary Medicine, University College Dublin, Dublin 4, Ireland

Edited by George Seidel, Colorado State University, and approved January 6, 2010 (received for review December 3, 2009)

Although genetically identical for autosomal Chrs (Chr), male and female preimplantation embryos could display sex-specific transcriptional regulation. To illustrate sex-specific differences at the mRNA level, we compared gene-expression patterns between male and female blastocysts by DNA microarray comparison of nine groups of 60 bovine in vitro-produced blastocysts of each sex. Almost one-third of the transcripts detected showed sexual dimorphism (2,921 transcripts; false-discovery rate, $P < 0.05$), suggesting that in the absence of hormonal influences, the sex Chrs impose an extensive transcriptional regulation upon autosomal genes. Six genes were analyzed by qPCR in in vivo-derived embryos, which displayed similar sexual dimorphism. Ontology analysis suggested a higher global transcriptional level in females and a more active protein metabolism in males. A gene homolog to an X-linked gene involved in network interactions during spliceosome assembly was found in the Y-Chr. Most of the X-linked-expressed transcripts (88.5%) were up-regulated in females, but most of them (70%) exhibited fold-changes lower than 1.6, suggesting that X-Chr inactivation is partially achieved at the blastocyst stage. Almost half of the transcripts up-regulated in female embryos exhibiting more than 1.6-fold change were present in the X-Chr and eight of them were selected to determine a putative paternal imprinting by gene expression comparison with parthenogenetic embryos. Five (*BEX*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) exhibited a higher expression in females than in parthenotes, suggesting that they are predominantly expressed by the paternal inherited X-Chr and that imprinting may increase the transcriptional skew caused by double X-Chr dosage.

gender | preimplantation | microarray | imprinting | X-inactivation

In mammals, sexual dimorphism is mostly attributable to sex-related hormonal differences in fetal and adult tissues; however, this may not be the sole determinant. Before gonad differentiation occurs, male and female preimplantation embryos display phenotypic differences that can only be attributed to the different sex Chr dosage. Although male and female blastocysts carry the same autosomal DNA, gender-specific transcription or translation occurs. At these early stages, sex Chrs modulate the genome machinery leading to differences in epigenetic status (1) and expression level of both X-linked (2, 3) and autosomal genes (1, 4, 5). These molecular events are reflected in phenotypic differences reported under some culture conditions, including differences in speed of embryo development, survival after vitrification, cell number at the blastocyst stage, and metabolism. In particular, glucose metabolism is thought to differ between male and female embryos (6), which may lead to a skewing in sex ratio because of preferential loss of embryos of one sex occurring both in vitro (7, 8) and in vivo (9, 10).

In a genomic context, transcriptional analyses during preimplantation development provide a useful tool to study hormone-independent sexual dimorphism phenomena. Both sex Chrs encode transcripts, which not only can have a direct effect upon phenotypic differences (i.e., *G6PD* and *HPRT*) but also can modulate the expression of autosomal genes (1). In adult tissues, one of the X-Chrs is inactivated, but some genes can escape from the X-Chr inactivation process and be expressed biallelically. This situation is especially common during preimplantation development, when X inactivation is a reversible dynamic process (11) and may lead to an up-regulation of X-linked genes in female embryos (2, 3). On the other hand, male embryos only contain the maternally inherited X-Chr; thus, an X-linked gene up-regulation in females may also occur as a result of an imprinting mechanism leading to a total or partial maternal allele transcriptional repression (11).

Global gene expression analyses in preimplantation embryos are scarce, mainly because of the technical difficulties in obtaining the necessary large number of embryos per group. To our knowledge, there is only one report on global differences in gene expression in male and female blastocysts, which used a transgenic mouse model to obtain the biological material (12). Sex-sorted semen constitutes a powerful tool for these studies, as it can provide a large number of embryos of known sex in species with a longer preimplantation period, such as bovine. In this study, we aimed to analyze preimplantation sexual dimorphism mechanisms at the transcriptional level by microarray gene-expression profiling of bovine blastocysts produced in vitro. We also confirmed the findings for in vivo-derived embryos, performed ontology analysis, reported a previously unreported Y-linked transcript, and determined the putative imprinting of eight X-linked genes up-regulated in female embryos.

Results

Microarray Overall Results and Validation. More than 1,000 blastocysts of known sex were produced in 12 independent experiments. The global gene-expression pattern of nine pools each of male and female bovine blastocysts ($n = 60$ blastocysts per pool) was compared with the GeneChip Bovine Genome Array. A total of 9,322 transcripts were present at the blastocyst stage. The total number of transcripts differing between male and female embryos is listed in Table 1 and Table S1. Hierarchical distribution clearly grouped the samples according to the sex, irrespective of the bull used (Fig. 1*A* and Fig. S1). Therefore, statistical analysis was performed by grouping the data from the nine arrays of each sex obtained from samples of the three different bulls. Principal component analysis demonstrated that the sexes clearly separated and, interestingly, within each sex, the three replicates from each bull clustered together (Fig. 1*A* and Fig. S1). The large sample size obtained after grouping the data allowed us to detect small

Author contributions: D. Rizos, P.L., and A.G.-A. designed research; P.B.-A. performed research; D. Rizos, D. Rath, and P.L. contributed new reagents/analytic tools; P.B.-A., D. Rath, and A.G.-A. analyzed data; and P.B.-A., P.L., and A.G.-A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Raw data from microarray experiments was submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo). The platform ID is GPL2112. The accession ID for the set of experiments described is GSE17921. A new 1382 pb cDNA was identified YZRSR2 (GQ426330).

[1]To whom correspondence should be addressed. E-mail: agutierr@inia.es.

This article contains supporting information online at www.pnas.org/cgi/content/full/0913843107/DCSupplemental.

Table 1. Results of microarray analysis of transcripts differentially expressed between male (M) and female (F) bovine blastocysts

Comparison (# of samples)	Multiple test correction	Up-regulated genes in females	Up-regulated genes in males	Total	Fold-changes > 2 female/male
F vs. M(9 vs. 9)	None	1,667	2,089	3,745	53/2
	FDR$P < 0.05$	1,330	1,591	2,921	53/2
	FDR $P < 0.01$	897	914	1,811	53/2
	Bonferroni $P < 0.05$	290	92	382	45/2

Statistical analysis from comparison of nine pools each of male and female embryos.

absolute differences and after false-discovery rate (FDR) assessment of significance ($P < 0.05$) correction was applied to reduce the number of false-positives, a total of 2,921 transcripts differed between male and female blastocysts, which constitutes almost one-third of the transcripts actively expressed. The fold-change for most of the transcripts was below 2. For 55 transcripts, the fold-change was higher than 2, 53 of which were up-regulated in females. Transcripts up-regulated (with the higher values) in male and female bovine blastocysts are listed in Tables S2 and S3. A higher level of fold-changes of up-regulated transcripts was found in female than in male embryos.

Array validation by quantitative PCR (qPCR) was performed in embryos produced with unsorted sperm to confirm that observed sex-related transcript differences were not artifacts of the use of sorted sperm. Eight X-linked genes (*BEX1*, *CAPN6*, *FMR1NB*, *SAT1*, *BEX2*, *X24112*, *SRPX2*, and *UBE2A*), one transcript putatively present on both sex Chrs (*Y2467*), a previously unreported gene located on the Y-Chr (*YZRSR2*), and four autosomal genes (*GSTM3*, *PGRMC1*, *LAMA1*, and *DNMT3A*), together with two Y-linked genes with an X-linked homolog not present on the array were analyzed. Fold-change values obtained by qPCR were very similar to those obtained in the array (Table 2).

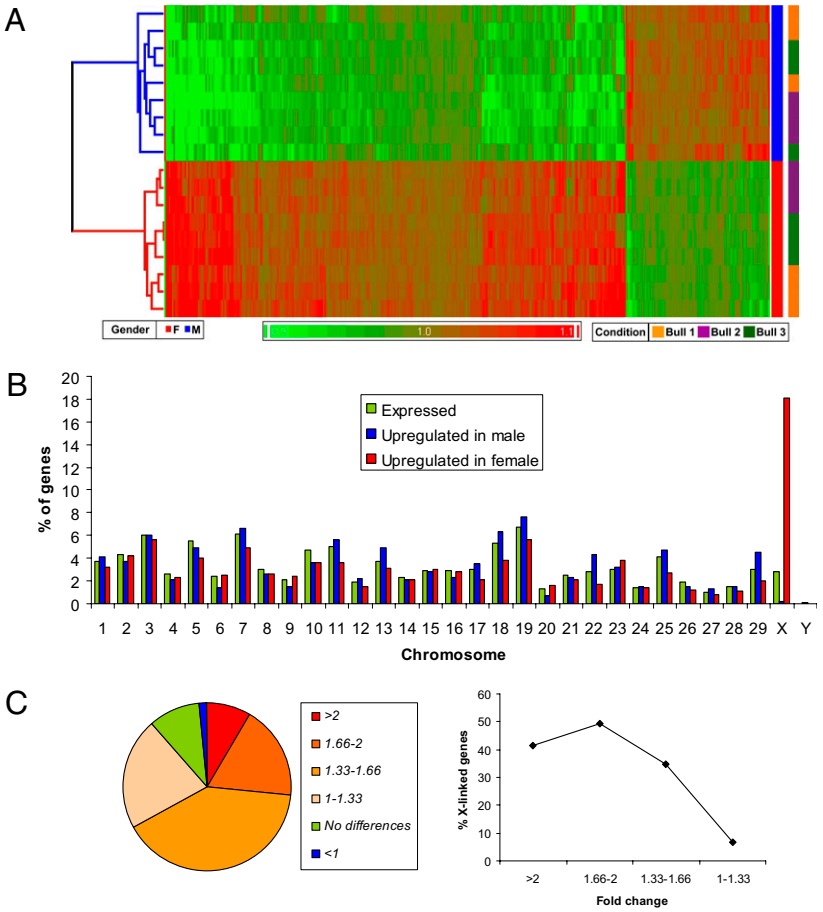


Fig. 1. Comparison of male and female bovine blastocysts. (*A*) Hierarchical clustering of the 382 differentially expressed transcripts (Bonferroni correction) between male and female bovine blastocysts, comparing three different bulls and the three pools of embryos derived from Y- and X-sorted semen from each bull. The color gradient determines normalized gene expression of all of the samples. The dendrogram on the left depicts the grouping of samples based on the similarity between them. Samples were clearly grouped according to sex (blue and red bars on *Left*). (*B*) Chr distribution for the total transcripts present (green bars) and up-regulated in male (blue bars) or female (red bars) embryos (FDR $P < 0.05$). Percentages for each Chr out of the total transcripts with a known Chr location (7,691, 1,287, and 1,065 transcripts for present and up-regulated in males and females, respectively). (*C*) The pie chart shows the percentage of expressed X-linked transcripts ($n = 218$) grouped according to the fold change (FDR $P < 0.05$ correction). From pink to red were up-regulated in females ($n = 193$), blue were up-regulated in males ($n = 3$), and green did not show differences ($n = 22$). The line chart shows the percentage of X-linked transcripts compared to the total up-regulated transcripts in females with a known location after FDR for four groups according to the fold-change.

Table 2. Validation of array data by real-time qRT-PCR analysis

Gene	qPCR*	Array*	Statistical correction
BEX1	5.6	4.04	Bonferroni $P < 0.05$
CAPN6	4.13	4.07	Bonferroni $P < 0.05$
GSTM3	3.52	3.94	Bonferroni $P < 0.05$
FMR1NB	3.02	3.1	Bonferroni $P < 0.05$
SAT1	2.83	2.73	Bonferroni $P < 0.05$
BEX2	2.32	2.8	Bonferroni $P < 0.05$
X24112	2.28	2.63	Bonferroni $P < 0.05$
SRPX2	1.75	2.43	FDR $P < 0.01$
PGRMC1	1.52	1.62	Bonferroni $P < 0.05$
UBE2A	2.56	1.78	Bonferroni $P < 0.05$
YZRSR2	–1†	–12.01	Bonferroni $P < 0.05$
DDX3Y	–1†		Not in array
EIF2S3Y	–1†		Not in array
Y2467	–5.38	–2.25	Bonferroni $P < 0.05$
LAMA1	–1.76	–1.93	Bonferroni $P < 0.05$
DNMT3A	–1.75	–1.19	FDR $P < 0.01$

*Expression fold-change of female versus male (positive values for up-regulated genes in females).
†Expressed only in males.

To determine whether the sex-related differences also occur for in vivo-derived embryos, the expression level of six genes was analyzed in in vivo-derived male or female embryos. Fold-change values obtained by qPCR were also similar to those obtained in the array (Fig. 2*A*).

Chr Distribution of Differentially Regulated Genes. Chr distribution comparison between expressed genes and up-regulated genes after FDR ($P < 0.05$) correction is shown in Fig. 1*B*. χ^2 analysis was performed to test for significant differences in Chr-location frequency between the three groups. The only Chr that displayed significant differences between up-regulated transcripts in males and females and expressed genes was the X-Chr, which accounted for 18.1% of the total up-regulated genes in females, whereas only 2.8% of the expressed transcripts were X-linked. Of the 218 X-linked transcripts expressed, 193 (88.5%) were up-regulated in females. Among them, only 10% exhibited a fold-change greater than 2, and most of them (70%) exhibited a fold-change lower than 1.66 (Fig. 1*C*, pie chart). Furthermore, X-linked genes accounted for almost half (47%) of the transcripts up-regulated in females that exhibit a fold-change higher than 1.66, whereas this percentage decreased (14%) in the groups with a lower fold change (Fig. 1*C*, line chart). The Chr distribution of the expressed X-linked genes along the X-Chr is shown in Fig. S2. Three regions, located at 0 to 3, 21 to 24, and 54 to 57 Mb account for 42.9% of the total transcripts up-regulated in females. No relation was found between fold-change and transcript location for the up-regulated X-linked transcripts in females and the 3 transcripts up-regulated in males and the 22 showing no sex-related differences were distributed in a similar way to the transcripts up-regulated in females. Some X-linked genes showing no sex-related dimorphism or up-regulation in males had a Y-Chr homolog, which suggests that the lack of sex-related differences may be caused by the transcription of both X- and Y-Chrs.

Gene Ontology. Gene ontology classification (FatiGo) was used for categorizing embryo expressed sequence tags of the gene ontology annotated genes present after FDR $P < 0.01$ correction (Fig. 3). Under the Molecular Function heading, nucleotide binding, DNA binding, nucleic acid binding, calcium ion binding, enzyme inhibitors, and transcription-factor activity were overrepresented among the up-regulated genes in female blastocysts, whereas hydrolase activity followed the opposite tendency. Biological Function analysis showed that transcription-related functions (regulation of transcription DNA-dependent, transcription, RNA splicing, and RNA processing) were overrepresented in genes up-regulated in females, whereas translation, proteolysis, and protein transport followed the opposite tendency. In addition, signal transduction, cell differentiation, and multicellular organismal development were overrepresented in females, whereas metabolic process and cell cycle were in males. Finally, under Cellular Component, mitochondrion, mitochondrial inner membrane, and ribosome were overrepresented among the up-regulated genes in males, whereas cytoplasm, extracellular region, cytoskeleton, and cell projection were overrepresented in females.

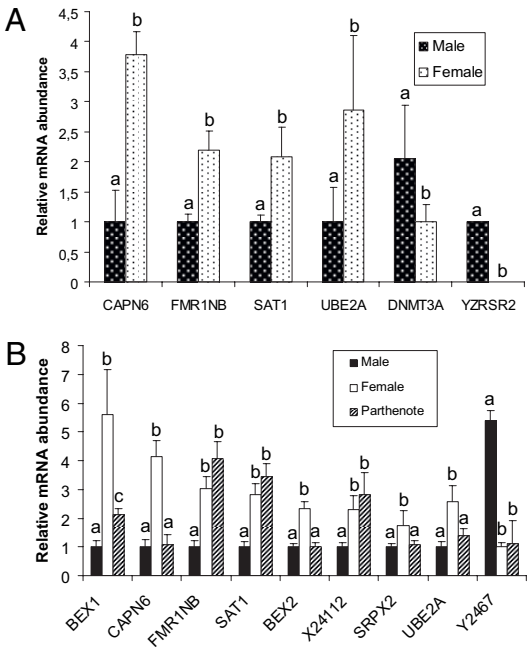


Fig. 2. Relative mRNA abundance. (*A*) Relative poly(A) mRNA abundance of six genes (four X-linked -*CAPN6*, *FMR1NB*, *SAT1* and *UBE2A*-, one Y-linked –*YZRSR2*-, and one autosomal -*DNMT3A*-) for male and female in vivo–derived blastocysts. (*B*) Relative poly(A) mRNA abundance of eight putative paternally expressed imprinted X-linked genes and one gene located on both X- and Y-Chrs (*Y2467*) for male (black bars), female (white bars), and parthenogenetic (dashed bars) in vitro blastocysts. Different letters indicate significant differences between groups based on one-way ANOVA ($P \leq 0.05$).

Identification of a Previously Unreported Y-Linked Transcript. In the microarray there were three probes that were theoretically complementary to the X-linked *ZRSR2*; however, only one of the probes showed differential expression between male and female blastocysts, and it showed the highest difference among all of the genes. For this reason we designed primers specifically to amplify this sequence to sequence and clone the complete cDNA. A previously unreported 1382 pb cDNA was identified (GQ426330). The sequence [*YZRSR2*, Y-linked zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2 homologous] has 100% homology with a recently published BAC present in the bovine Y-Chr (AC216982.4) and is homologous to the X-linked gene *ZRSR2* (87% homology with the putative bovine gene -XR_028361.2- and 84% for human -BC113480.1-). The sequence contains an ORF encoding a protein of 446 amino acid residues. The transcript was found to be uniquely expressed in male embryos and tissues and was expressed from the beginning of embryonic genome activation (16-cell stage) onward and in all of the male tissues analyzed except the spleen (Tables S4 and S5).

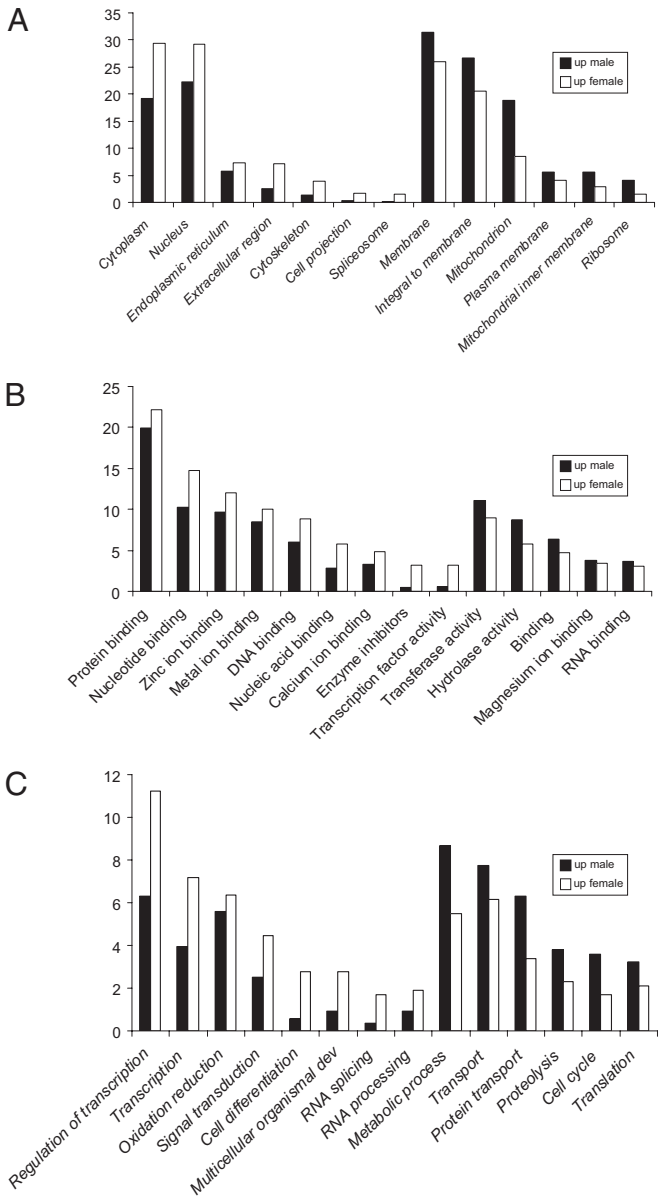


Fig. 3. Fatigo-based comparative analysis of gene ontology for the family of genes exhibiting sex-related transcriptional differences based on (*A*) molecular function, (*B*) biological function, and (*C*) cellular component.

Putative Imprinted Gene Analysis. The expression of eight X-linked genes that were up-regulated in female blastocysts was analyzed in male and female blastocysts produced with unsorted semen and in parthenogenetic blastocysts. The expression level of five genes (*BEX1*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) differed between parthenogenetic and female blastocysts, suggesting that the paternal allele is expressed at a higher level than the maternal (Fig. 2*B*). The expression level of one transcript present in both Y- and X-Chrs (*Y2467*) did not differ between parthenogenetic and female blastocysts. The presence of transcripts of these nine genes was also analyzed in denuded oocytes, preimplantation stage embryos, and adult tissues (Tables S4 and S5). All genes were present in oocytes and blastocysts, but the transcript presence in other stages differed among genes with two of them (*SAT1* and *UBE2A*) present in all stages. *CAPN6*, *SAT1*, and *UBE2A* were present in all tissues analyzed, whereas *Y2467* was not found in adult tissues, *FMR1NB* was only expressed in testes, and *SRPX2* in kidney. The genes with a known location are shown in Fig. S2.

Discussion

Gene-expression variation may play a significant role in gender-specific early embryo development disparity through up- or down-regulating genes within physiological pathways. Herein we report extensive transcriptional differences occurring during pre-implantation and therefore not attributable to sex-specific hormonal actions. The transcription of genes located on the sex Chrs exerts direct or indirect effects on biological processes, which lead to sexual dimorphism. Genes on the Y-Chr will be only present in males, and X-Chr effects will be mediated by sex differences in the dose of X genes or their parental imprint. Almost half of the up-regulated transcripts in female embryos exhibiting a fold-change higher than 1.66 were X-linked, similar to the situation observed in mice (12). Dosage compensation by random X-Chr inactivation ensures an equal transcription level of X-linked genes for both sexes. Nevertheless, some genes may escape from X-Chr inactivation processes and be expressed from both X-Chrs, which cause an up-regulation in females. This escape is especially common during the preimplantation period (2, 3), when X inactivation is a reversible dynamic process (11) and leads to a phenotypic sexual dimorphism (7, 8). Although in vivo- and in vitro-produced embryos have been found to display the same transcriptional sexual dimorphism (12), sex-related differences may be exaggerated in in vitro-produced embryos compared with those derived in vivo, as X-Chr inactivation seems to be disrupted by some in vitro conditions (13, 14). However, the six genes (four X-linked, one Y-linked, and one autosomal) analyzed showed similar sex-related transcriptional differences for in vivo- or in vitro-derived blastocysts, suggesting that X-Chr inactivation is only partially achieved at the blastocyst stage in both in vivo and in vitro conditions, and that our in vitro conditions did not increase the sexual dimorphism. Sex-related differences are likely to be higher under sub-optimal conditions, which are known to alter sex ratio by preferential loss of embryos of one sex (7, 8). A similar situation occurs for other sex-related phenotypic differences, such as speed of development, which has been reported to be altered under some in vitro culture conditions, which lead to sex ratio distortion (15). However, under the conditions used in this study, no difference was observed in speed of development between male and female embryos (16), and the percentage of blastocysts at day 7 were similar between both groups (17).

Four-fifths of the up-regulated genes in female embryos and almost all of the up-regulated genes in male embryos were autosomal genes, indicating that sex-Chrs can exert an extensive regulation on the transcription of some autosomal genes, and therefore indirectly modulate sexual dimorphism. Gene ontology analysis was in agreement with observations previously reported. Among the cellular components, mitochondria and mitochondria inner membrane were overrepresented in the up-regulated genes in males, which is in agreement with the higher mtDNA copy number found in male embryos (1). Spliceosome, nucleotide, DNA, nucleic acid and calcium-ion binding, transcription factors activity, transcription and its regulation, and RNA splicing and processing were overrepresented among the up-regulated genes in females, which suggests a higher global transcriptional level for this sex; this is in accordance with the lower expression of de novo DNA methyltransferases (Dnmts) and lower methylation status reported in female embryos (1, 18) and stem cells (19). These differences in DNA-methylation regulation may account for the embryonic sex-specific susceptibility to a methyl-deficient maternal diet, and thus lead to gender-specific long-term effects in the offspring (20). The higher transcriptional level found in females is unlikely to be the direct consequence of the presence of an actively transcribed X-Chr in females instead of the Y-Chr, as X-Chr accounted for just 2.8% of the total expressed transcripts. How-

ever, it has been suggested that X-Chr encodes a modifier locus whose product represses de novo Dnmts, which could indirectly lead to these differences (19). In contrast, among the up-regulated genes in male blastocysts, ribosome, translation, proteolysis, and protein transport were overrepresented, suggesting a more active protein metabolism, which may be related with the differences in amino acid turnover recently found between male and female bovine blastocysts (21). Finally, cell differentiation, signal transduction, and development were overrepresented in females, which may suggest differences in developmental processes.

All these phenotypic sex-related differences cannot be attributable to sex-specific hormonal differences but to genomic actions, which are not only restricted to the preimplantation period, as sexual dimorphism phenomena independent of hormonal interaction have been reported in adult tissues. Thus, a large sex effect on gene expression (30% of expressed genes) and trans-regulation was described in mouse macrophages that were cultured 2 weeks ex vivo, and thus were not influenced by endogenous sex steroids (22). Furthermore, the chromosomal sex of muscle-derived stem cells influences their ability to promote skeletal muscle regeneration by differential transcription of genes related to cell stress response (23). Moreover, male and female cells, independent of hormonal influences, respond differently to stressors, and a possible sex-dependent gene regulatory mechanism was suggested to explain sexually dimorphic physiology and pathology (24). Interestingly, male and female embryos of different species (mice, bovine, and human) respond differentially to environmental stress (25). Collectively, these data suggest that a large degree of sexually dimorphic gene expression may be directly dependent on X- and Y-Chr dosage, rather than on the hormonal environment, and that cells differ and respond to stress innately according to sex, irrespective of their history of exposure to sex hormones.

The unique Y-linked transcript described here (*YZRSR2*) is located in the nonrecombining portion of the Y-Chr, which only maintains those genes responsible for large fitness effects, generally being restricted to those either required for male function or those that determine sex (26). In both humans and mice, *ZRSR2* has an autosomal homolog (*ZRSR1*) (27), which in mice is a paternally expressed imprinted gene that is silenced during oogenesis (28). The function of *YZRSR2* remains unclear, but it seems to have evolved from the X-linked gene *ZRSR2*, which encodes an essential splicing factor (29). Thus, *YZRSR2* may have a role in sex determination and sexual dimorphism by operating at the posttranscriptional level, although more studies would be needed to test this hypothesis. The Y-Chr is particularly important for the study of sex determination and fertility because of its rapid species-specific differential evolution and divergence (30). Unfortunately, it is the only Chr that remains unsequenced in the cattle genome project (31).

The higher expression of X-linked genes in female compared to male embryos may be caused by a preferential paternal allele expression (12), by a double allele expression, (2, 13, 14) or by a combination of both. In the first case, a lower expression for male and parthenogenetic embryos compared with their female counterparts is expected, as they lack a paternal X-Chr (12, 32). Five genes (*BEX1*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) were found to be preferentially expressed by the paternal allele (Fig. 2*B*). Maternal allele expression level was lower, but not absent. Although autosomal and X-linked imprinted genes can be biallelically expressed at the blastocyst stage, which may mask imprinting mechanisms, they may show quantitative effects of imprinting in terms of differences in gene expression between monoparental and biparental embryos (11, 32). Most of the X-linked genes up-regulated in female embryos exhibited a fold-change lower than 2, suggesting that X-Chr inactivation occurred partially. Unfortunately, very little is known about X-chromosome inactivation during preimplantation development in other species than mouse (33). In adult tissues, X-chromosome inactivation is a random and stable process, although some paternally expressed X-linked genes, such as *XIST* and *RHOX5* (12), have been reported (http://igc.otago.ac.nz/home.html). Furthermore, females with Turner syndrome (XO) differ in their cognitive and behavioral phenotypes, according to the parental origin of their single X chromosome in human and mice (34), which may be caused by imprinting. Imprinting mechanisms have been proposed to evolve from a mechanism to defend the genome against transposable elements and, compared to autosomal chromosomes, the X chromosome has generated a disproportionately high number of functional retroposed genes in mammalian species (35).

We do not know if these differences in gene expression are similar in other species. However, in one study on mouse embryos, using a microarray analysis with 20,371 transcripts and three pools of male and female blastocysts, fewer than 600 sex-biased genes were detected (12). The large degree of sex bias in gene expression that we detected in bovine blastocysts can partly be attributed to the large power we had to detect highly significant sex effects on gene expression, even when the absolute effect was small because of the large sample size (nine pools each of male and female blastocysts). In our study, when only one bull and three pools each of male and female bovine blastocysts were analyzed, a small number of sex-biased genes was detected because of the small sample size and the low power to detect small effects as significant.

The high number of genes exhibiting sexual dimorphism shows an extensive transcriptional regulation lead by the sex Chrs and provides a basis for the study of transcriptional sexual dimorphism without hormonal interaction, which occurs both for in vivo- and in vitro-derived embryos. Furthermore, they suggest that data from male and female embryos should be analyzed separately in gene expression analyses focused on individual blastocysts and embryos produced by nuclear transfer from a male or female cell line, on embryo-maternal gene communication, and on stem cell lines. In addition, we have identified five X-Chr linked genes whose expression level in female embryos differs from male and parthenogenetic embryos, suggesting that they are predominantly expressed by the paternally inherited X-Chr and opening the possibility of a synergistic effect of imprinting and double-X dosage on X-linked genes transcriptional sex-related differences. Future analysis of gene expression in in vivo embryos produced under different environmental conditions may help to understand the nature and consequences that these early sex differences may have on sex ratio control in mammals. By analyzing these early sex differences it may be possible to exert greater control on sex ratio manipulation in domestic animals and to better understand other aspects of early embryo development, early sex determination mechanisms, X inactivation, and epigenetic and genetic processes related to early development that may have a short-term effect on implantation and a long-term effect on offspring.

Materials and Methods

Sperm Collection, Sorting, and Verification of the Sorting Procedure. Semen was collected and sorted flow cytometrically for sex from each of three bulls as previously described (36). Approximately 90% of the embryos produced with sorted sperm were of the predicted sex (17).

Embryo Production. In vitro fertilization procedures were performed as previously described (36). Details in *SI Materials and Methods*. In vivo-derived blastocysts were obtained from superovulated cows on day 7 after artificial insemination. Details in *SI Materials and Methods*.

RNA Isolation and Target Preparation. Total RNA was isolated and purified from 18 pools of 60 day 7 blastocysts each (three bulls, two sexes, three replicates) using the RNeasy Micro kit (Qiagen). Embryos were pooled randomly to avoid batch-to-batch variation. Labeled cRNA was synthesized with a linear RNA amplification method (Message Amp Premier Kit, Ambion) following the manufacturer's instructions and using 50 ng of total RNA as template for reverse transcription.

Array Hybridization and Scanning. Labeled cRNA was fragmented in fragmentation buffer [5× buffer: 200 mM Tris-acetate (pH 8.1)/500 mM KOAc/150 mM MgOAc] and hybridized to the microarrays (n = 18 arrays, as described above) in 200 μL of hybridization solution containing 15 μg labeled target in 1× Mes buffer (0.1 M Mes/1.0 M NaCl/20 mM EDTA 0.01%/Tween20) and 0.1 mg/mL herring sperm DNA, 10% DMSO, 0.5 mg/mL BSA, 50 pM control oligonucleotide B2 and 1× eukaryotic hybridization controls (bioB, bioC, bioD, cre). Both control oligonucleotide B2 and eukaryotic hybridization controls were purchased from Affymetrix. The hybridization mix was applied to the GeneChip Bovine Genome Array (Affymetrix), which contains 24,072 bovine gene probe sets, representing more than 23,000 transcripts, including assemblies from 19,000 UniGene clusters. Arrays were placed on a rotisserie and rotated at 0.4 × g for 16 h at 45 °C. Following hybridization, the arrays were washed and stained with a streptavidin-phycoerythrin conjugate (Molecular Probes). The arrays were scanned using a confocal scanner (GC3000_Affymetrix). The image data were analyzed by GeneChip Operating Software (GCOS 1.4, Affymetrix).

Array Bioinformatic and Statistical Analysis. For the bioinformatic analysis dChip and Affy/AffyPLM (Bioconductor) software were used to detect outlier samples and Partek Genomics Suite 6.4 (Partek software, Partek Inc.) to perform gene expression analysis. RMA processing was used for normalizing the data as well as a global median normalization. The change in expression of each gene was calculated by determining the fold-change (ratio) of the mean intensity of each group. Statistical analysis was based on a regression model and ANOVA to look for significant genes between conditions and hierarchical un/supervised analysis performed using Average linkage and Euclidean distance for classifying the samples. Bonferroni or FDR corrections were applied to reduce the total number of false-positives. Chr distribution was performed based on the Entrez link provided in annotation file Bovine.na29.annot.csv available on the Affymetrix Web site among the 9,322 expressed transcripts. Gene ontology (FatiGO: http://babelomics.bioinfo.cipf.es) was used for categorizing embryo expressed sequence tags with respect to gene function, including molecular function, biological process, and cellular component (37). Raw data from microarray experiments was submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo). The platform ID is GPL2112. The accession ID is GSE17921.

Independent Verification of Array Data Using Real-Time RT-PCR. Poly(A) RNA was extracted after sexing from individual blastocysts produced in vitro with unsorted semen or by parthenogenetic activation or in vivo with unsorted semen following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) with minor modifications (17). (Details are available in *SI Materials and Methods*). The quantification of all mRNA transcripts was carried out by real-time qRT-PCR following a previously described protocol (1). Five pools of cDNA per experimental group, each obtained from 10 in vitro-produced or 5 in vivo-derived embryos, were used with two repetitions for all genes of interest. Details of protocol for quantification of mRNA transcripts are available in *SI Materials and Methods*). The primers listed in Table S6 were used to amplify specific fragments referring to the selected transcripts. PCR fragments were sequenced to verify the resulting PCR product.

YZRSR2 Cloning and Sequencing. Reverse transcription was performed in blastocyst RNA and full-length cDNA of YZRSR2 was cloned with 5′- and 3′-RACE using SMART Technology (Clontech Laboratories, Inc.).

ACKNOWLEDGMENTS. This work was supported in part by Grants AGL2009-11358 (to A.G.-A.), AGL2009-11810 (to D. Rizos), and FPU (to P.B.-A.) from the Spanish Ministry of Science and Innovation, by Science Foundation Ireland (P.L.).

1. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol Genomics* 32:264–272.
2. Gutierrez-Adan A, Oter M, Martinez-Madrid B, Pintado B, De La Fuente J (2000) Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. *Mol Reprod Dev* 55:146–151.
3. Taylor DM, et al. (2001) Quantitative measurement of transcript levels throughout human preimplantation development: analysis of hypoxanthine phosphoribosyl transferase. *Mol Hum Reprod* 7:147–154.
4. Larson MA, Kimura K, Kubisch HM, Roberts RM (2001) Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-tau. *Proc Natl Acad Sci USA* 98: 9677–9682.
5. Morton KM, et al. (2007) Altered mRNA expression patterns in bovine blastocysts after fertilisation in vitro using flow-cytometrically sex-sorted sperm. *Mol Reprod Dev* 74:931–940.
6. Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA (1991) Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. *J Reprod Fertil* 93:125–132.
7. Bredbacka K, Bredbacka P (1996) Glucose controls sex-related growth rate differences of bovine embryos produced in vitro. *J Reprod Fertil* 106:169–172.
8. Gutierrez-Adan A, Granados J, Pintado B, De La Fuente J (2001) Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. *Reprod Fertil Dev* 13: 361–365.
9. Davis D, Gottlieb M, Stampnitzky J (1998) Reduced ratio of male to female births in several industrial countries: a sentinel health indicator? *JAMA* 279:1018–1023.
10. Helle S, Laaksonen T, Adamsson A, Paranko J, Huitu O (2008) Female field voles with high testosterone and glucose levels produce male-biased litters. *An Behav* 75: 1031–1039.
11. Mak W, et al. (2004) Reactivation of the paternal X chromosome in early mouse embryos. *Science* 303:666–669.
12. Kobayashi S, et al. (2006) Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. *Curr Biol* 16:166–172.
13. Wrenzycki C, et al. (2002) In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol Reprod* 66:127–134.
14. Nino-Soto MI, Basrur PK, King WA (2007) Impact of in vitro production techniques on the expression of X-linked genes in bovine (bos taurus) oocytes and pre-attachment embryos. *Mol Reprod Dev* 74:144–153.
15. Xu KP, Yadav BR, King WA, Betteridge KJ (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. *Mol Reprod Dev* 31:249–252.
16. Rizos D, Bermejo-Alvarez P, Gutierrez-Adan A, Lonergan P (2008) Effect of duration of oocyte maturation on the kinetics of cleavage, embryo yield and sex ratio in cattle. *Reprod Fertil Dev* 20:734–740.
17. Bermejo-Alvarez P, Lonergan P, Rath D, Gutierrez-Adan A, Rizos D (2010) Developmental kinetics and gene expression in male and female bovine embryos produced *in vitro*. *Reprod Fertil Dev* 22:426–436.
18. Gebert C, et al. (2009) DNA methylation in the IGF2 intragenic DMR is re-established in a sex-specific manner in bovine blastocysts after somatic cloning. *Genomics* 94:63–69.
19. Zvetkova I, et al. (2005) Global hypomethylation of the genome in XX embryonic stem cells. *Nat Genet* 37:1274–1279.
20. Sinclair KD, et al. (2007) DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci USA* 104:19351–19356.
21. Sturmey RG, et al. (2010) Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol Reprod Devel* 77:285–296.
22. Bhasin JM, et al. (2008) Sex specific gene regulation and expression QTLs in mouse macrophages from a strain intercross. *PLoS One* 3:e1435.
23. Deasy BM, et al. (2007) A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *J Cell Biol* 177:73–86.
24. Penaloza C, et al. (2009) Sex of the cell dictates its response: differential gene expression and sensitivity to cell death inducing stress in male and female cells. *FASEB J* 23:1869–1879.
25. Gutierrez-Adan A, et al. (2006) Developmental consequences of sexual dimorphism during pre-implantation embryonic development. *Reprod Domest Anim* 41 (Suppl 2):54–62.
26. Skaletsky H, et al. (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 423:825–837.
27. Nabetani A, Hatada I, Morisaki H, Oshimura M, Mukai T (1997) Mouse U2af1-rs1 is a neomorphic imprinted gene. *Mol Cell Biol* 17:789–798.
28. Zhang Z, et al. (2006) Comparative analyses of genomic imprinting and CpG island-methylation in mouse Murr1 and human MURR1 loci revealed a putative imprinting control region in mice. *Gene* 366:77–86.
29. Tronchere H, Wang J, Fu XD (1997) A protein related to splicing factor U2AF35 that interacts with U2AF65 and SR proteins in splicing of pre-mRNA. *Nature* 388:397–400.
30. Graves JA (2006) Sex chromosome specialization and degeneration in mammals. *Cell* 124:901–914.
31. Elsik CG, et al. (2009) The genome sequence of Taurine cattle: a window to ruminant biology and evolution. *Science* 324:522–528.
32. Latham KE, Rambhatla L (1995) Expression of X-linked genes in androgenetic, gynogenetic, and normal mouse preimplantation embryos. *Dev Genet* 17:212–222.
33. Patrat C, et al. (2009) Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. *Proc Natl Acad Sci USA* 106:5198–5203.
34. Davies W, Isles AR, Wilkinson LS (2005) Imprinted gene expression in the brain. *Neurosci Biobehav Rev* 29:421–430.
35. Emerson JJ, Kaessmann H, Betran E, Long M (2004) Extensive gene traffic on the mammalian X chromosome. *Science* 303:537–540.
36. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? *Biol Reprod* 79:594–597.
37. Fernandez-Gonzalez R, et al. (2009) Analysis of gene transcription alterations at the blastocyst stage related to the long-term consequences of in vitro culture in mice. *Reproduction* 137:271–283.

Supporting Information

Bermejo-Alvarez et al. 10.1073/pnas.0913843107

SI Material and Methods

In Vitro Embryo Production. Selected cumulus–oocyte complexes (COCs) obtained from ovaries collected at slaughter were matured for 24 h in TCM-199, supplemented with 10% (vol/vol) FCS (FCS) and 10 ng/mL epidermal growth factor at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. In vitro fertilization was performed with X- or Y-sorted sperm from three different bulls or unsorted semen from one of the bulls (Bull 1) as described in ref. 1. Matured COCs were inseminated with 12.5 μL of frozen-thawed, percoll-separated sperm added to 25-μL droplets under mineral oil (15–20 oocytes per droplet) at a final concentration of 1×10^6 spermatozoa/mL. Gametes were co-incubated at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. At ≈20 h postinsemination, presumptive zygotes were denuded and transferred to 25-μL culture droplets (1 embryo per microliter) under mineral oil. Blastocysts were produced in 12 independent experiments (equals days of ovary collection). In all cases, X- and Y-sperm from an individual bull were used on the same day and were never split across days. Depending on the availability of oocytes, one, two, or all three bulls were processed on a given day. Details for developmental rates and sexing accuracy of the bulls used in this study can be found in ref. 2. Parthenogenetic activation was achieved by incubation of mature oocytes in 5 μM ionomycin for 5 min followed by 3 h incubation in 2 mM 6-DMAP. Embryo culture took place in SOF + 5% FCS. Plates were incubated for 7 days at 39 °C under an atmosphere of 5% CO_2, 90% N_2, and 5% O2 with maximum humidity. Day-7 blastocysts produced from X- and Y-sorted sperm were snap-frozen in groups for microarray analysis and those produced with unsorted sperm and parthenogenetic activation had their zona pellucida removed with 0.2% pronase and were then individually snap-frozen for array validation. Oocytes and embryos collected at different stages were snap-frozen in groups of 10 to analyze the evolution of transcription during preimplantation stages of different genes presented in the array.

In Vivo Embryo Production. In vivo bovine blastocysts were produced using standard superovulation protocol. Briefly, the estrous cycles of crossbred beef heifers were synchronized using a combination of an intravaginal progesterone device (CIDR) and administration of a prostaglandin F2α analog to ensure luteolysis. Beginning on day 10 of the synchronized cycle, each animal received twice daily injections of Folltropin (Bioniche Animal Health) for 4 days, with prostaglandin given with the sixth injection. Animals were inseminated at estrus and embryos were recovered by flushing the reproductive tract at slaughter. Blastocysts were snap-frozen individually in liquid nitrogen for subsequent analysis.All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin.

Independent Verification of Array Data Using Rea-lTime RT-PCR. Poly (A) RNA was extracted from individual blastocysts produced in vitro with unsorted semen or by parthenogenetic activation or derived in vivo following the manufacturer's instructions, using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) with minor modifications. After 5 min incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet, suspended in washing buffer A and stored at 4 °C while DNA extraction and sexing was performed. The DNA present in lysis buffer was extracted with phenol/chloroform treatment and finally suspended in 16 μL of milliQ water. Eight microliters of each sample were used to perform embryo sexing by PCR as described in ref. 1. After embryo sexing, the individually stored poly(A) RNA from 10 (in vitro) or 5 (in vivo) embryos of the same sex or parthenotes were pooled and RNA extraction continued. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline, Ecogen) using poly(T) primer, random primers, and MMLV reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were then incubated at 42 °C for 60 min to allow the reverse transcription of RNA, followed by 70 °C for 10 min to denature the RT enzyme.

The quantification of all mRNA transcripts was carried out by real-time qRT-PCR; five groups of cDNA per experimental group, each obtained from 10 embryos, were used with two repetitions for all genes of interest. PCR was performed by adding a 2-μL aliquot of each sample to the PCR mix containing the specific primers to amplify histone H2Az (H2a.z), brain-expressed X-linked 1 (BEX1), calpain 6 (CAPN6), GST Mu3 (GSTM3), fragile-X mental retardation 1 neighbor (FMR1NB), spermidine/spermine N1-acethyltransferase 1 (SAT1), brain-expressed X-linked 2 (BEX2), sushi-repeat-containing protein X-linked 2 (SRPX2), progesterone receptor membrane component 1 (PGRMC1), ubiquitin-conjugating enzyme E2A (UBE2A), DEAD box polypeptide 3 Y-linked (DDX3Y), eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (EIF2S3Y), laminin alpha 1 (LAMA1), DNA-methyltransferase 3 alpha (DNMT3A), and three nonannotated transcripts present in the array and named X24112, YZRSR2 and Y2467. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table S6. For quantification, real-time PCR was performed as previously described (3). qPCR conditions were 94 °C for 3 min followed by 35 cycles (94 °C 10 s, 56 °C 30 s, 72 °C 10 s, and 10 s of fluorescence acquisition – SYBR-) (Rotor Gene 6000, Corbett Research). Each pair of primers were tested to achieve efficiencies close to 1 and then the comparative cycle threshold (CT) method was used to quantify expression levels as described (4). To avoid primer-dimmers artifacts, fluorescence was acquired in each cycle at a temperature higher than the melting temperature of primer-dimmers (specific for each product, 80–86 °C). Then, the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. When the efficiency is close to 1, within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the endogenous control (H2a.z) CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as a constant to subtract from all other ΔCT sample values. Fold-changes in the relative gene expression of the target were determined using the equation $2^{-\Delta\Delta CT}$.

Expression Analysis on Preimplantation Stages and Tissues. RNA extraction and DNA synthesis of denuded oocytes and preimplantation embryos was performed as described above. Male and female adult tissues (brain, medulla, testes, spleen, kidney, lung, and

muscle) were collected from a local slaughterhouse and immediately snap-frozen in liquid nitrogen. RNA extraction was performed with ULTRASPEC (Ecogen) avoiding DNA contamination by DNase treatment following the protocol described in ref. 4 with minor modifications. PCR was performed and products were visualized in 2% ethidium bromide stained gels under UV light.

1. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? *Biol Reprod* 79:594–597.
2. Bermejo-Alvarez P, Lonergan P, Rath D, Gutierrez-Adan A, Rizos D (2010) Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. *Reprod Fertil Dev* 22:426–436 .
3. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol Genomics* 32:264–272.
4. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.

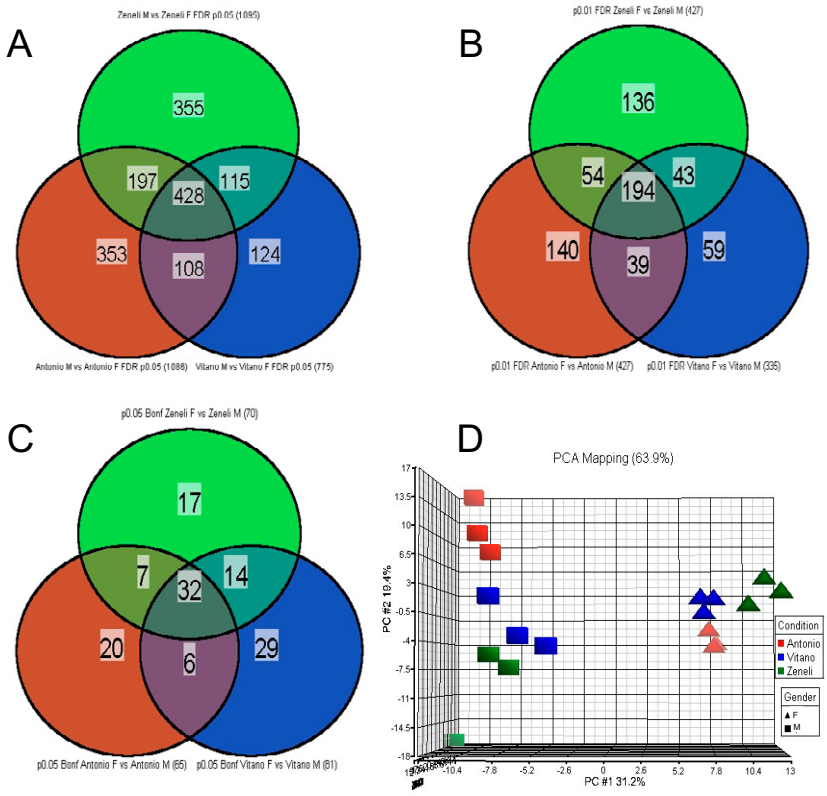


Fig. S1. Analysis of microarray data for male and female bovine blastocysts between the three individual bulls. Venn diagrams showing the number of transcripts common or specific to each bull comparison using three statistical corrections: (*A*) False-discovery rate (FDR) at $P < 0.05$; (*B*) FDR at $P < 0.01$; and (*C*) Bonferroni at $P < 0.05$. (*D*) Principal component analysis mapping of the 18 analyzed samples showing the aggregation of the samples according to the sex and to the bull.

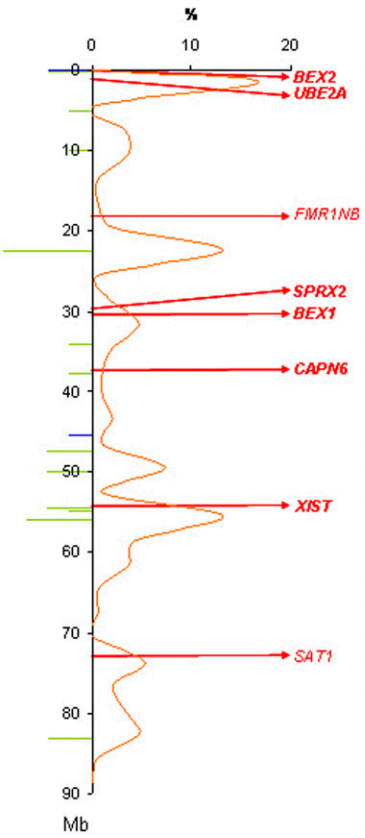


Fig. S2. Chromosome distribution of the expressed X-linked transcripts. The position along the X-chromosome is shown on a Megabase vertical scale. For representation, the chromosome was divided into 30 3-Mb length sections and a scatter chart on the right shows the percentage of X-linked transcripts up-regulated in females located on each section compared with the total X-linked transcripts up-regulated in females with known location. The location of the genes analyzed for putative imprinting plus XIST are noted by red arrows (*Right*); gene names in bold letters are preferentially or totally paternally expressed. (*Left*) Color bars depict the location of X-linked genes showing no differences between sexes (n = 22, green bars) or being up-regulated in male (n = 3, blue bars).

Table S1. Results of microarray analysis of transcripts differentially expressed at the blastocyst stage between male and female bovine blastocysts

Comparison (# of samples)	Multiple test correction	Up-regulated transcripts in females	Up-regulated transcripts in males	Total	Fold-changes > 2 female/male
Bull 1: F vs. M (3 vs. 3)	NO	1,050	1,347	2,397	67/4
	FDR $P < 0.05$	567	528	1,095	61/3
	FDR $P < 0.01$	293	134	427	53/2
	Bonferroni	57	13	70	14/1
Bull 2: F vs. M (3 vs. 3)	NO	1,095	894	1,989	58/1
	FDR $P < 0.05$	529	246	775	56/1
	FDR $P < 0.01$	273	62	335	41/1
	Bonferroni	74	7	81	17/1
Bull 3: F vs. M (3 vs. 3)	NO	1,058	1,295	2,353	64/6
	FDR $P < 0.05$	555	531	1,086	59/6
	FDR $P < 0.01$	277	149	426	46/4
	Bonferroni	53	12	65	16/3

Statistical analysis from comparison of three bulls and three pools each of male and female embryos per bull.

Table S2. Transcripts up-regulated in female bovine blastocysts

Fold-change	Gene symbol	Description	Map position	Affymetrix number
5.69697	*XIST*	X (inactive)-specific transcript	X	Bt.11847.1.A1_at
4.81758	*LOC617729*	Similar to germ cell less		Bt.19174.1.A1_at
4.07092	*CAPN6*	Calpain 6	X	Bt.10150.1.S1_at
4.03808	*BEX1*	Brain expressed, X-linked 1	X	Bt.16678.1.S1_at
3.94148	*GSTM3*	GST M3 (brain)	3	Bt.5304.1.S1_at
3.1044	*FMR1NB*	Fragile X mental retardation 1 neighbor	X	Bt.17305.1.A1_at
3.01817	*MGC140151*	Hypothetical protein LOC783451		Bt.3698.1.S1_at
2.80938	*BEX2*	Brain expressed X-linked 2	X	Bt.4818.1.S1_at
2.79954	*NGFRAP1 /// WBP5*	WW domain binding protein 5 /// nerve growth factor receptor (TNFRSF16) associat		Bt.1998.1.S1_at
2.77223	*LOC618696*	Similar to trophoblast Kunitz domain protein 5	13	Bt.26365.1.A1_at
2.72556	*SAT1*	Spermidine/spermine N1-acetyltransferase 1	X	Bt.7594.1.S1_at
2.67507	*HSPA1A*	Heat shock 70kDa protein 1A	23	Bt.5154.1.S1_s_at
2.62714	*LOC511106*	Serpin peptidase inhibitor, clade B like	24	Bt.9693.1.S1_at
2.62576	—	Transcribed locus	X	Bt.24112.1.A1_at
2.46584	*RNASE1*	Ribonuclease	10	Bt.4630.1.S1_at
2.44574	*PAGE4*	P-antigen family, member 4 (prostate associated)	X	Bt.6375.1.S1_at
2.44426	—	CDNA clone IMAGE:8067330		Bt.3722.1.S1_at
2.42561	*SRPX2*	Sushi-repeat-containing protein, X-linked 2	X	Bt.8109.2.A1_at
2.42269	*LOC786521*	Similar to growth differentiation factor 3	5	Bt.26222.1.A1_at
2.40878	*NUDT10*	Nudix (nucleoside diphosphate linked moiety X)-type motif 10	X	Bt.24396.3.A1_at
2.39311	*RESP18*	Regulated endocrine-specific protein 18	2	Bt.1107.1.S1_at
2.35584	*LDOC1*	Leucine zipper, down-regulated in cancer 1	X	Bt.16830.1.S1_at
2.33049	*HSPA1A*	Heat shock 70kDa protein 1A	23	Bt.5154.1.S1_at
2.28281	*MATN4*	Matrilin 4	13	Bt.15484.2.A1_at
2.27552	*PHLDA2*	Pleckstrin homology-like domain, family A, member 2	29	Bt.1702.1.A1_at
2.27285	*DHRS9*	Dehydrogenase/reductase (SDR family) member 9	2	Bt.12764.1.S1_at
2.26844	*UCHL1*	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	6	Bt.5408.1.A1_at
2.23403	—	Transcribed locus, moderately similar to XP_228803.4 PREDICTED: similar to spind		Bt.6921.1.S1_at
2.22899	*UBD*	Ubiquitin D	23	Bt.5897.2.S1_at
2.2128	*HSPA1A*	Heat shock 70kDa protein 1B	23	Bt.23161.2.A1_at
2.20333	*BDH2*	3-hydroxybutyrate dehydrogenase, type 2	6	Bt.1792.1.S1_at
2.18206	*LOC527068*	Aldo-keto reductase family 1 member C3 -like	13	Bt.23094.5.S1_at
2.17899	*MAOB*	Monoamine oxidase B	X	Bt.22460.1.S2_at
2.16263	*MAGEH1*	Melanoma antigen family H, 1	X	Bt.27079.1.S1_at
2.15041	*LGALS3BP*	Lectin, galactoside-binding, soluble, 3 binding protein	19	Bt.6597.1.S1_at
2.11523	*CLU*	Clusterin	8	Bt.12504.1.S1_at
2.10778	*APOL3*	Apolipoprotein L, 3	5	Bt.28313.1.S1_at
2.09222	—	Transcribed locus		Bt.8707.1.S1_at
2.0887	*PRPS1*	Phosphoribosyl pyrophosphate synthetase 1	9	Bt.3500.1.S1_at
2.07758	*BRWD3*	Bromodomain and WD repeat domain containing 3	X	Bt.26037.1.A1_at
2.0607	*LOC783344*	Similar to armadillo repeat containing, X-linked 5	X	Bt.18144.1.A1_at
2.06029	*TUBA4A*	Tubulin, alpha 4a	2	Bt.5183.1.S1_at

Table S2. Cont.

Fold-change	Gene symbol	Description	Map position	Affymetrix number
2.05841	—	Transcribed locus		Bt.17129.1.A1_at
2.02324	*TRAPPC2*	Trafficking protein particle complex 2	X	Bt.27952.1.S1_at
2.00277	*TUBA4A*	Tubulin, alpha 4a	2	Bt.5183.2.S1_at
1.98973	*UPK1A*	Uroplakin 1A	18	Bt.337.1.S1_at
1.97291	*UBQLN2*	Ubiquilin 2		Bt.26593.1.A1_at
1.96544	—	Transcribed locus, moderately similar to NP_006112.3 keratin 1 [Homo sapiens]		Bt.879.1.S1_at
1.9635	*ZAP70*	Zeta-chain (TCR) associated protein kinase 70kDa	11	Bt.20905.1.S1_at
1.96167	*SULT1A1*	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	25	Bt.3537.1.S1_at
1.95339	*THEM4*	Thioesterase superfamily member 4	3	Bt.10185.1.S1_at
1.94875	*PDK3*	Pyruvate dehydrogenase kinase, isozyme 3	X	Bt.28108.1.S1_at
1.94866	—	Transcribed locus		Bt.16164.2.A1_at
1.94588	*TNNI3*	Troponin I type 3 (cardiac)	18	Bt.11438.1.S1_at
1.93171	*MGC127538*	Hypothetical protein MGC127538		Bt.2577.1.S1_at
1.92109	*RNF113A*	Ring finger protein 113A		Bt.9525.1.A1_at
1.91648	*SNX12*	Sorting nexin 12	X	Bt.6730.1.S1_at
1.91203	*TCEAL8*	Transcription elongation factor A (SII)-like 8		Bt.4822.1.A1_at
1.9115	*TM4SF1*	Transmembrane 4 L six family member 1	1	Bt.6087.1.S1_at
1.90139	—	Transcribed locus		Bt.29559.1.A1_at
1.90074	—	Transcribed locus		Bt.27388.1.A1_at
1.89922	*MSL3L1*	Male-specific lethal 3-like 1 (Drosophila)	X	Bt.26659.1.S1_at
1.89498	—	Transcribed locus		Bt.9959.3.S1_a_at
1.88554	*SLITRK2*	SLIT and NTRK-like family, member 2	X	Bt.17460.1.A1_at
1.88486	—	—		Bt.24184.1.A1_at
1.87044	*MID1IP1*	MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))	X	Bt.11034.1.S1_at
1.8673	*STEAP2*	Six transmembrane epithelial antigen of the prostate 2	4	Bt.22045.1.S1_at
1.86095	*CITED1*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyl-terminal domain,	X	Bt.4437.1.S1_at
1.84912	*LY6G6C*	Lymphocyte antigen 6 complex, locus G6C	23	Bt.9737.1.S1_at
1.84314	*HSPB8*	Heat shock 22kDa protein 8	17	Bt.22526.1.S1_at
1.83629	—	Transcribed locus		Bt.22686.1.S1_at
1.8359	*ABHD4*	Abhydrolase domain containing 4	10	Bt.28090.1.S1_at
1.82804	*ATRX*	Alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevi	X	Bt.8265.1.A1_at
1.82472	—	Transcribed locus		Bt.7322.1.S1_at
1.82433	*CXCL16*	Chemokine (C-X-C motif) ligand 16	19	Bt.22009.1.S1_at
1.81427	*LOC534630; LOC613705*	Similar to RNA binding motif protein, X-linked 2; Y3388 transposase	X	Bt.9546.2.S1_a_at
1.8142	*DYNLT3*	Dynein, light chain, Tctex-type 3		Bt.1844.1.S1_at
1.80754	*MCTS1*	Malignant T cell amplified sequence 1	X	Bt.9940.1.S1_at
1.80692	*S100A4*	S100 calcium binding protein A4	3	Bt.5493.1.S1_at
1.80566	*ALDOC*	Aldolase C, fructose-bisphosphate	19	Bt.4837.1.A1_at
1.80129	—	—		Bt.20724.1.S1_at
1.79894	*FAM122B*	Family with sequence similarity 122B	X	Bt.24200.1.S1_at
1.79516	—	Transcribed locus		Bt.6445.1.S1_at
1.79079	*TSPAN6*	Tetraspanin 6		Bt.3821.1.A1_at
1.78802	*LOC613674*	Hypothetical protein LOC613674	X	Bt.19984.1.S1_at
1.78683	*CETN2*	Centrin, EF-hand protein, 2	X	Bt.2559.1.S1_at
1.78002	*UBE2A*	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	X	Bt.13063.1.S1_at
1.77614	*MAGED2*	Melanoma antigen family D, 2	X	Bt.5078.1.S1_at
1.77427	*WDR44*	WD repeat domain 44	X	Bt.216.1.S1_at
1.77413	*MAP3K12*	Mitogen-activated protein kinase kinase kinase 12	5	Bt.29962.1.S1_at
1.76628	*APEX2*	APEX nuclease (apurinic/apyrimidinic endonuclease) 2	X	Bt.1184.1.S1_at
1.76603	—	Transcribed locus, strongly similar to NP_001091853.1 retinitis pigmentosa GTPas		Bt.10928.1.A1_at
1.76491	*S100A11*	S100 calcium binding protein A11 (calgizzarin)	3	Bt.3750.1.S1_at
1.75419	*NKRF*	NFKB repressing factor		Bt.2958.1.A1_at
1.75237	*FUNDC1*	FUN14 domain containing 1	X	Bt.18513.1.S1_at

All transcripts for which the change in expression level is higher than 1.75-fold are listed (Bonferroni corrections).

Table S3. Transcripts up-regulated in male bovine blastocysts

Fold-change	Gene symbol	Description	Map position	Affymetrix number
−12.0163	*YZRSR2*	Y-linked zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2	Y	*Bt.24372.1.S1_at*
−2.25315	—	Transcribed locus		*Bt.2467.1.A1_at*
−1.93739	*LAMA1*	Laminin, alpha 1	24	*Bt.26511.1.S1_at*
−1.75692	*BPHL*	Biphenyl hydrolase-like (serine hydrolase; breast epithelial mucin-associated)	23	*Bt.1663.1.A1_at*
−1.70267	*P2RX4*	Purinergic receptor P2X, ligand-gated ion channel, 4	17	*Bt.15997.1.S1_at*
−1.67361	*SLC6A20*	Solute carrier family 6 (proline IMINO transporter), member 20	22	*Bt.28747.1.S1_at*
−1.63797	—	Transcribed locus		*Bt.13701.1.A1_at*
−1.55877	—	Transcribed locus		*Bt.11733.1.S1_at*
−1.55343	—	Transcribed locus		*Bt.5998.1.S1_at*
−1.54639	*SHPK*	Sedoheptulokinase	19	*Bt.14637.1.A1_at*
−1.53131	—	Transcribed locus		*Bt.22747.1.S1_at*
−1.52509	*LOC512529*	Hypothetical LOC512529	5	*Bt.11226.1.S1_at*
−1.4575	—	Transcribed locus		*Bt.9413.1.S1_at*
−1.45683	—	CDNA clone IMAGE:8277899		*Bt.9612.1.S1_at*
−1.44552	*ABCB6*	ATP-binding cassette, subfamily B (MDR/TAP), member 6	2	*Bt.14310.1.S1_a_at*
−1.44319	*HSD17B8*	Hydroxysteroid (17-beta) dehydrogenase 8	23	*Bt.2187.1.S1_at*
−1.44193	*LOC788567*	Hypothetical protein LOC788567	29	*Bt.12080.1.S1_at*
−1.433	*PRMT7*	Protein arginine methyltransferase 7	18	*Bt.21927.1.S1_at*
−1.43021	—	Transcribed locus, moderately similar to NP_060272.3 HEAT repeat containing 2 [H		*Bt.22403.1.S1_at*
−1.42759	*SNAPC5*	Small nuclear RNA activating complex, polypeptide 5, 19kDa	10	*Bt.13422.1.A1_at*
−1.42671	—	Transcribed locus		*Bt.9848.1.A1_a_at*
−1.42644	*HN1L*	Hematological and neurological expressed 1-like	25	*Bt.10127.1.S1_at*
−1.42401	*LOC516579*	Similar to Probable phospholipid-transporting ATPase IIA (ATPase class II type 9	13	*Bt.21546.1.S1_at*
−1.42319	*COQ6*	Coenzyme Q6 homolog, monooxygenase (*S. cerevisiae*)	10	*Bt.22142.2.S1_a_at*
−1.41898	*LOC525138*	Similar to CG3337-PA	20	*Bt.2552.1.S1_at*
−1.41637	*FBXL6*	F-box and leucine-rich repeat protein 6	14	*Bt.2511.1.S1_at*
−1.41156	*SPLL2B*	Signal peptide peptidase-like 2B	7	*Bt.12223.1.S1_at*
−1.41114	*LOC514296*	Hypothetical LOC514296	22	*Bt.8874.1.S1_at*
−1.40872	*LOC524279*	Similar to LPS responsive and Beige-like anchor protein LRBA	17	*Bt.13411.1.S1_at*
−1.40651	—	Transcribed locus		*Bt.23606.2.S1_at*

All transcripts for which the change in expression level is lower than −1.4-fold are listed (Bonferroni corrections).

Table S4. Gene expression during preimplantation development of eight candidate-imprinted genes present on the X-chromosome, one putatively X- and Y-linked, and one Y-chromosome linked

Gene	Oocyte	4–8 cell	16 cell	Morula	Blastocysts
YZRSR2	No	No	Yes	Yes	Yes
Y2467	Yes	Weak	No	Weak	Yes
BEX1	Yes	No	No	Yes	Yes
CAPN6	Yes	No	No	Yes	Yes
FMR1NB	Yes	No	Yes	Yes	Yes
SAT1	Yes	Yes	Yes	Yes	Yes
BEX2	Yes	No	No	No	Yes
X24112	Yes	Weak	No	No	Yes
SRPX2	Yes	No	No	Yes	Yes
UBE2A	Yes	Yes	Yes	Yes	Yes

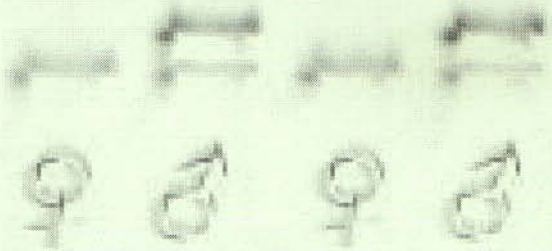
Table S5. Gene expression in different tissues of adult animals of eight candidate-imprinted genes present on X-chromosome, one putatively X- and Y-chromosome linked, and one Y-chromosome linked

Gene	Brain	Medulla	Testes	Spleen	Kidney	Lung	Muscle
YZRSR2	Yes	Yes	Yes	No	Yes	Yes	Yes
Y2467	No	No	No	No	No	No	No
BEX1	Yes	Weak	Yes	No	No	No	No
CAPN6	Yes	Yes	Yes	Yes	Yes	Yes	Yes
FMR1NB	No	No	Yes	No	No	No	No
SAT1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
BEX2	Yes	Yes	Yes	Weak	Yes	Yes	No
X24112	No	No	Yes	Yes	No	No	No
SRPX2	No	No	No	No	Yes	No	No
UBE2A	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table S6. Details of primers used for qRT-PCR

Gene	Primer sequence (5′–3′) (Forward/Reverse)	Fragment size, bp	GenBank accession no.
H2AFZ	AGGACGACTAGCCATGGACGTGTG/CCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
BEX1	ACCTAGAGGAAATCGCAGACGG/AAGCATCTTGCCATGAAACAGG	434	NM_001077034
CAPN6	TTTTAAATGATTGCGTGCTGAAC/AAAGCCACATCCTCCATGAAGC	135	AU278104.1
GSTM3	ATCGCCCGCAAGCACAATATG/TCCAGGCACTTGGGCTCAAAC	290	NM_001046560.1
FMR1NB	TTTCCAACAACATGCGTTCCAG/TTCCTCATCCACTGCTGTTTCC	363	NM_001102262.1
SAT1	GCCAGGTTGCAATGAAGTGTCG/AAACATGCAACAACGCCACTGG	320	NM_001034333.1
BEX2	AGAACCTATGGCCCTCCTTTCG/AAGCTGGTAATAGGAAACGCG	409	NM_001077087.1
X24112	TACAGCTGACCAAACTAAAGGC/TGCTTAGCTTCTGAGTTTGGAC	204	CK846762
SRPX2	ACTCGCTCCTACTTCAACATG/AGAGACTTGCTTGAGGAAAGG	337	NM_001014926.1
PGRMC1	TGTGTGTCACAAATCCAGAAAG/AATCATGCAGTTAGGTCAATCG	265	NM_001075133.1
UBE2A	GGGCTCCGTCTGAGAACAACATC/CATACTCCCGCTTGTTCTCCTGG	336	XM_864331
YZRSR2	CAGGAGGAAGAACAAGAGACAAAC/CTTCTTCACTGTACTCTAAGT	396	GQ426330
DDX3Y	AGTAGCAATCGTGGCCGTTCTAGG/CCTGAGAGCTGTAATTTCCG	217	XM_001251699.1
EIF2S3Y	TGTTAAGAAAATTCCAGTACCTCTC/ATACTGAAGATCATTGTGTTCC	275	FJ627276.1
Y2467	GGACTAACGTACAATCTACCTC/AAGGCACGGTCTCCTCTTAGTC	554	CB460806
LAMA1	CCCTGCCAGCAATGCACACATC/TCGGATGCCGTTCTGTTGAAGG	341	AF010231
DNMT3A	CTGGTGCTGAAGGACTTGGGC/CAGAAGAAGGGGCGGTCATC	317	AY271299

GENERAL DISCUSSION/ DISCUSIÓN GENERAL



GENERAL DISCUSSION

Evaluation of the putative spermatozoa selection by the oocyte

Two possible groups of mechanisms may be involved in the sex ratio skews originated before conception. The first one involves a putative spermatozoa selection by the oocyte which could alter the equality in fertilization chances between X- or Y-bearing spermatozoa. The second is based on putative intrinsic differences between X- or Y-bearing spermatozoa which could affect the equal chances for X- and Y-bearing spermatozoa to reach the fertilization site. In chapters 1 and 2, the feasibility of the first hypothesis was tested.

Spermatozoa sex selection mechanisms by the oocyte have been proposed to explain the relation between sex ratio and three different features of the oocyte: stage of maturation [1-3], testosterone concentration in the follicular fluid [4] and the side of the ovary of origin [5, 6]. A putative spermatozoa sex selection affected by the maturation stage of the oocyte provides an explanation for the influence of the time of insemination in relation to ovulation upon sex ratio suggested for several species, including bovine (discussed in introduction). It has been suggested that delaying insemination allows metaphase II-arrested oocytes to process Y sperm more effectively [1-3]. In chapter 1, the effect of the maturation stage of the oocyte upon sex ratio was examined, and maturation time was found to affect kinetics of first cleavage and cleavage rate, as discussed in the chapter, but no effect was found in sex ratio.

The existence of a putative spermatozoa selection by the oocyte would has a great impact on sex-sorted sperm technology, as it will imply that fertility rates will be low irrespective of the advance in the sorting techniques [7] (discussed in introduction). In chapter 2, we tested the hypothetical spermatozoa selection by the oocyte by examining the differences in in vitro fertility between unsorted, sorted (X- or Y-sorted) and sorted/recombined (a mix of X- and Y-sorted) sperm. If the oocyte is able to select the sperm, a decrease in fertility would be obtained for both sorted groups (X- and Y-sorted), whereas fertility would improve for the sorted/recombined

group. A decrease in fertility was found for all three groups fertilized with sorted semen compared with unsorted, which is contrary to a putative spermatozoa selection.

Three studies carried out after the publication of chapter 2 have confirmed the absence of a selective process of X- or Y-bearing spermatozoa in the oocyte [8-10]. The first used fluorescent *in situ* hybridization to determine the percentage of Y-bearing spermatozoa in epididimal murine sperm and in spermatozoa attached to *in vivo* matured murine oocytes, and found no skew from the theoretical 1:1, concluding that murine oocytes do not selectively attract one class of spermatozoa [8]. This finding is consistent with a previous study which found equal numbers of X- and Y-bearing bovine spermatozoa between those which penetrate the bovine ooplasm [11]. The other two studies address the effect of testosterone upon the putative oocyte sex-selection mechanisms [4]. The first one found that increasing testosterone concentrations in the maturation media do not affect sex ratio [9], whereas the second failed to repeat the findings of the study published by Grant *et al.* [10]. A testosterone-dependent sperm selection mechanism by the oocyte would provide a physiological explanation for the relation between maternal dominance and sex ratio reported in several species (discussed in introduction). However, it has not been reported a relation between high serum testosterone levels, which are related to maternal dominance, and high follicular testosterone levels.

The side of the ovary an oocyte comes from has been suggested to affect the likelihood of that oocyte from being fertilized from and X- or Y-bearing spermatozoa. In this sense, sex ratio has been reported to differ from left or right uterine horn in several species where uterine migration is low (discussed in introduction). If this controversial notion is true, spermatozoa selection by the oocyte is not the only possible explanatory mechanism: X- or Y-bearing spermatozoa chances to reach the fertilization site could be different depending of the uterine side. In agreement, a recently published paper which observed different sex ratios in right and left bovine uterine horns, did not found skewed sex ratios neither in unilaterally-ovariectomized heifers nor in embryos derived from IVF from abattoir derived oocytes originated from right or left ovaries [5, 12].

The results presented in chapter 2 constitute strong evidence against the existence of a selection of X- or Y-bearing sorted spermatozoa by the oocyte. This hypothetical mechanism requires the presence of sex-specific proteins in the membrane of the spermatozoa which would allow the cumulus-oocyte complex to distinguish them, which is theoretically possible, as explained in the introduction. However, several studies have failed to detect those sex specific antigens [13-15]. Furthermore, this mechanism could imply a reduction in fertility, as in *in vivo* fertilization, very few spermatozoa reach the fertilization site, where a selection mechanism would reduce the chance for fertilization. Contrary to the oocytes, which are present in reduced number, each insemination provides millions of spermatozoa, which made a spermatozoa selection along the female genital tract more feasible than an oocyte selection mechanism. Taken together, these findings propose intrinsic differences between X- or Y-bearing spermatozoa which could affect the equal chances for X- and Y-bearing spermatozoa to reach the fertilization site as responsible for preconceptual sex selection mechanisms. These oocyte-independent mechanisms could explain possible preconceptual effects of different female parameters on sex ratio, such as time of insemination, testosterone level or body condition.

Analysis of the low fertility obtained with sorted semen

As described in the introduction, the use of sorted semen is frequently associated with low fertility rates both in *in vivo* and *in vitro* conditions, which prevents its use in conventional animal production systems. This reduction in fertility may be caused by differences in sperm quality (spermatozoa damage during the sorting procedure and lower doses of spermatozoa) or by a putative oocyte selection mechanism. As above mentioned, the last is inconsistent with the findings of chapter 2. Moreover, in spite of the use of the same concentration of live spermatozoa selected by Percoll method, a reduction in fertility was observed for sorted sperm in chapters 2 and 3. As review in Chapter 3, the deleterious effect on spermatozoa caused by sperm sorting procedure is responsible for the reduced fertility rates.

The farm data available show an increased percentage of cows returning to estrus at normal inter-estrus intervals after insemination with sorted semen [16]. This situation may be caused by a reduction of the fertilization ability of the sorted semen

or by an increase in embryonic mortality prior to the maternal recognition. To discriminate between both possibilities, cleavage rate at different time points and blastocyst yield was recorded in our *in vitro* study. A reduction in both parameters were noted in the groups fertilized with sorted semen, but a similar blastocyst/embryo cleaved early (before 33 hpi) ratio was obtained, suggesting that there was no increased embryo mortality, and that the reduction in fertility was due to a reduced fertilization ability. Furthermore, a delay in the timing of the first cleavage was obtained for sorted sperm, which may be associated with the reduced motility reported for sorted cryopreserved spermatozoa [17, 18]. This delay could be also caused by a longer time for spermatozoon processing by the oocyte following fertilization with sorted semen, as in mouse, ICSI with DNA fragmented (30 %) sperm leads to a 2 h delay in active male pronuclear demethylation [19]. However, the potential DNA damage of sperm sorted with both staining and laser illumination has been estimated to increase by 3.2 % compared with unsorted sperm [20], which may not have any consequence. Interestingly, the alteration in the evolution of first cleavage differed significantly between sorted semen from different bulls, which is consistent with the differences in fertility found between different bulls following IVF [21-23] or AI [24]. This finding suggests that susceptibility of sperm to the damage caused by the sorting procedure may differ between bulls, and IVF could provide a valuable tool to test these differences in susceptibility and, thus, allow sire selection to obtain higher fertility rates following AI or further improvements in the sorting procedure.

Damaged spermatozoa may be able to fertilize oocytes resulting in low-quality embryos [19], which may implant and undergo pregnancy resulting in detrimental long-term effects in the offspring [25], which will restrict seriously the use of sex-sorted technology in cattle and human. In chapter 3, a putative negative effect of the use of sorted semen on two embryo quality parameters (speed of development and gene expression) was analyzed. Neither timing of blastocysts appearance nor relative transcript abundance of genes related with apoptosis, detoxification of ultraviolet-induced oxygen radicals, pregnancy recognition, placenta formation and DNA methylation were affected by the use of sex-sorted sperm. These findings, together with the normalcy of the offspring obtained by sorted semen [26], suggest that sperm sorting do not affect embryo quality.

Sexual dimorphism in speed of development and survival in culture medium

Two different approaches were used to determine a possible sexual dimorphism in speed of development and survival in culture medium. Firstly, in chapter 1, embryos produced with unsorted semen were sexed at the 2-cell or blastocyst stage at different times of appearance. If a sexual dimorphism in terms of speed of development exists, changes of the sex ratio observed at different times of appearance are expected; whereas if a selective embryo loss of one determined sex occurs, different sex ratios between the 2-cell and the blastocyst stage should be observed. Secondly, in chapter 3, the evolution of first cleavage and blastocysts appearance was recorded for groups of embryos resulting from IVF with X- or Y-sorted semen (around 90 % of sexing accuracy). In this case, differences in the evolution of first cleavage and blastocysts appearance should be noted when using X- or Y-sorted semen from the same bull if sex-related differences in speed of development do exist; and differences in blastocyst/embryo cleaved ratio should be found between groups fertilized with X- or Y-sorted semen from the same bull, in the case of sex-specific survival rates. No sex-related differences in speed of development or embryo survival were noted in both approaches. As reviewed in the introduction, these sex-related differences may appear only under adverse conditions.

Analyzing the data from chapter 1-3, an increased sex ratio after IVF was noted. In chapter 1, the percentage of males at 48 hpi was 55.9 and 59.1 % for the 24 and 16 h maturation groups, respectively. Similarly, in chapter 2 the percentage of males in the 2- to 4-cell embryos sexed was around 57 % for both groups fertilized with unsorted and sorted-recombined sperm. Finally, an increased cleavage rate was obtained for Y-sorted semen compared with X-sorted in all three bulls analyzed. Taken together, these results suggest that the fertilization capacity of Y-bearing spermatozoa following IVF may be slightly higher than that of X-bearing spermatozoa. An increased secondary sex ratio has been reported in bovine with a 52.9 % of males over 500000 dairy cattle [27]. Similarly, in humans, although the percentage Y-chromosome bearing spermatozoa in semen is close to 50.3 %, live birth sex ratio in different countries is about 51.3 % [28]. Several statistic studies have confirmed that human sex ratio is about 106:100, with variations in different countries (reviewed in [29]). Furthermore, there is a higher loss for male fetuses than female

[30, 31]. Taken together, primary sex ratio has been proposed to differs greatly from the theoretically expected equality in human [29]. In agreement with this hypothesis, human embryonic sex ratio has been calculated to be 164:100 and to decrease to 111:100 at the fetal stage in Finish population [32].

Epigenetic and transcriptional preimplantation sexual dimorphism

Preimplantation sexual dimorphism studies may help in the understanding of the selective loss of embryos of a determined sex occurring under adverse conditions which are responsible for the sex ratio skews occurring after conception and linked to a reduction in fertility. Physiological differences observed under suboptimal *in vitro* conditions –in terms of speed of development, survival after vitrificacion and cell number (reviewed in introduction)- should have a molecular root. However, at the molecular level only few evidences, based on the transcription of some specific genes and glucose metabolism, have been reported.

Epigenetics is the basis for the transcriptional differences which modulates the translation that leads to functional differences. In chapter 4, relative mRNA abundance of genes related with the epigenetic regulation of transcription was found to differ between sexes. In particular, the two *de novo* DNA methyltransferases were highly expressed in male embryos, suggesting that the genome of the male embryos may be highly methylated, and thus more silenced [33] compared with female embryos. The finding of a higher methylation level in one of the five sequences analyzed, confirmed this hypothesis and suggests that sex-related differences in methylation are not a genome-wide but a sequence- or genomic region-specific phenomenon. Moreover, two enzymes related with histone methylation and therefore with genome silencing [33], were also highly transcribed in male embryos. In addition, the gene ontology analysis of chapter 5 suggests a higher global transcriptional level in female embryos, in agreement with the findings of chapter 4. After the publication of chapter 4, new evidence for sexual dimorphism in methylation status was provided in bovine. Gebert *et al.* found that the methylation level of one differentially methylated region (DMR) of the *IGF2* gene was lower in female than in male blastocyst [34]. Lower methylation levels have been also found in female EGCs and PGCs compared with male, but these differences may be caused by

the demethylating effect of the female genital ring (reviewed in [34]). However, in murine ES cells, which are directly derived from the blastocysts, XX cell lines were hypomethylated respect XY lines [35], and it has been suggested that the X chromosome may encode a modifier locus whose product represses *de novo* DNA methyltransferases [35]. Taking into account that in chapter 5 it was found that X-chromosome inactivation is far from being accomplished at the blastocyst stage, this locus is likely to be expressed at higher levels in females, which, thereby, will display reduced levels for these enzymes. Sexual dimorphism at the epigenetic level has been also found in neonatal mice, with higher levels of both histone methylation and acethylation in males [36]. Interestingly, the higher level of histone acethylation seemed to be caused by testosterone exposure, but the higher level of histone methylation could not be reach in females exposed to testosterone [36]. These sex-specific differences in methylation levels may lead to long-term effects, as it will be discussed below.

Telomere length was found to be shorter in male blastocysts (chapter 4). Telomere lengthening during early development is especially important, because the telomere reserves in newborns are established at the morula-blastocyst transition [37], and therefore, differences in telomere length at the blastocyst stage may have long term consequences. Consistently, immediately after birth, male telomeres have been reported to be shorter in mouse and rat [38, 39], and in the Xq arm in humans [40]. The establishment of a relation between this observation and the lower life expectance for male in several species including human [41] may be too speculative, as many other factors influence sex-specific mortality. The molecular mechanism responsible for this difference remains unclear, although a X-linked locus with a major effect on telomere length has been identified [42], and *de novo* DNA methyltransferases are negative regulators of telomere length [43].

Mitochondria are the most abundant organelles in the mammalian oocyte and early embryo [44] and have been demonstrated to play a critical role in preimplantation development [45-47], because they are essential for oxidative glucolysis, whose importance increases in the morula-blastocysts transition (reviewed in introduction and [48]). During preimplantation development, each mitochondrion contents a single copy of mtDNA, which avoids the quantification by qPCR [49].

Replication of mtDNA during preimplantation development has been proved in mouse and bovine [50, 51], which possibilities the existence of differences between sexes. Moreover, mtDNA has been hypothesized to be involved in sexual dimorphism [52]. In chapter 4, a higher content of mtDNA was observed in male embryos. Consistently, in chapter 5, gene ontology analysis showed that among the cellular components, mitochondria and mitochondria inner membrane were over-represented in the genes upregulated in males. The sexual dimorphism in the content of mtDNA may explain the differences in glucose metabolism and susceptibility to oxidative stress among male and female embryos [53].

As reviewed in the introduction, sex-related differences have been reported for the expression both sex chromosome- and autosomal-linked genes. However, with the exception of a microarray study carried out in the mouse model [54], all the studies have been based on a limited number of genes. In chapters 3 and 4, the previously unknown sexually dimorphic expression patterns for several genes (*DNMT3A*, *DNMT3B*, *HMT1*, *ILF3*, *GSTM3* and *PGRMC1*) were reported. Large scale analyses of the transcriptional differences are necessary to evaluate the extension of the preimplantation sexual dimorphism in gene expression and to validate the previous findings observed in studies which analyzed a small number of genes. In chapter 5, it was found that in the absence of hormonal differences, sex chromosomes impose an extensive transcriptional regulation upon autosomal genes, affecting to almost one third (2921) of the transcripts detected. The array was performed on *in vitro* derived embryos produced with sorted semen, but the validation was performed with both *in vitro* and *in vivo* derived embryos produced with unsorted semen. These findings have important implications for both developmental biology and genome regulation studies. Preimplantation embryos constitute an ideal model for the study of sexually dimorphic gene expression without hormonal influences. Our results are consistent with non-hormonal dependent sexual dimorphism phenomena found in adult cells (discussed in chapter 5), and suggest that a large degree of sexually dimorphic gene expression may be directly depend on sex chromosomes rather than on hormonal levels. The large preimplantation sexual dimorphism at the transcriptional level has implications in sex-specific embryo mortality, X-chromosome inactivation and long term effects, as it will be discussed below. Gene ontology analysis agreed with previous observations, such as the differences in epigenetic status and mtDNA.

Furthermore, protein metabolism related genes were overrepresented among the upregulated genes in male, which may play a role in the sexual dimorphism in aminoacid turnover recently found for bovine blastocysts [55]. Another important implication of the results presented herein is that when individual embryos are used for gene expression analysis, the male and female data should be analyzed separately, because the transcriptional differences found may not be caused by the treatment, but be a result of the sexual dimorphism. The same can be said when nuclear transfer embryos produced by one cell line, and thus being XX and XY, are compared with a pool of XX or XY IVF or *in vivo*-derived embryos.

Suboptimal environmental conditions during preimplantation development can originate long-term effects in the offspring due to alterations in the epigenetic reprogramming [25]. Long term effects have been found to be sex-specific in many cases, in agreement with the epigenetic differences observed in chapters 4 and 5. Probably, the most studied long term effect is the large offspring syndrome (LOS), which is caused by suboptimal *in vitro* culture conditions and it is characterized by a disproportionate growth of the fetus accompanied by a reduction in its viability [56]. The presence of serum in the culture media of mouse embryos is known to produce long-term effects similar to the LOS, which differ between male and female embryos; in particular increased body weight was only found in females and several behavioral abnormalities also depended on sex [57]. In the same animal, but using other model (presence or absence of a growth factor in the embryo culture medium), other study associated suboptimal *in vitro* culture conditions with increased body weight and decreased relative brain size in males, but not in females [58]. In humans, Beckwith-Wiedemann syndrome (BWS) is an epigenetic disorder originated at the preimplantation period which is considered to be the equivalent for the LOS, as it is characterized by similar alterations and has been related with altered methylation patterns in DMRs of genes involved in IGF2 signalling (such as hypomethylation in *KvDMR1*) [59]. Remarkably, BWS occurs at a relatively high frequency in monozygotic twins, and in almost all cases, the affected twins are female [60, 61], which fits with the higher methylation status found in male embryos in chapters 4 and 5 and in [34]. Furthermore, in sheep, a methyl-deficient maternal diet during the periconceptional period lead to long term effects in the offspring by affecting methylation levels of some *loci* in a sex-specific manner: over half of the affected *loci*

were specific to males, which was consistent with a greater clinically relevant phenotypic effect for that sex [62]. The differences in protein metabolism suggested in chapter 5 and in [55], may also lead to long-term effects. In rats, maternal low protein diet during the preimplantation period had gender-specific long term effects, such as reduced birth weight in female offspring and increased systolic blood pressure and abnormal organ/body mass ratios in male offspring [63].

X-chromosome inactivation in bovine blastocysts

To compensate the gradual loss of genes in the Y-chromosome across evolution, mammals developed the X-chromosome inactivation (XCI) mechanism. In eutherian mammals, X-chromosome inactivation occurs in the maternal or paternal X chromosome randomly, early in embryogenesis. Some studies consider that XCI is virtually complete in different adult tissues. The degree of completion of XCI varies among studies depending on the tissue [64], from 5.4 % of X-linked genes with increased female expression in human lymphoblastoid cell lines [65] to 15-25 % in fibroblasts and hybrid cells [66]. XCI was firstly suggested in 1961 to explain mosaic phenotypes seen in female mice heterozygous for sex-linked mutations in coat color genes [67] and then confirmed by later observations of heterozygous women for different X-linked genes, such as *G6PD*, *HPRT* and *PGK* [68-71]. In mouse embryos, the paternal X chromosome is fully active after EGA [72], then it undergoes imprinted inactivation from the cleavage states, and in the cells of the ICM of the expanded or hatching blastocyst it occurs a reversal of the inactive state, with a loss of epigenetic marks such as histone modifications or polycomb proteins, which give rise to the embryo-proper random XCI [73]. However, little is known about XCI during the preimplantation period in other species, and large variations among mammalian species may occur [74]. In chapter 5, we observed that in the bovine blastocyst, XCI is far from been accomplished. Consistently with the presence of both X-chromosomes partially active, most of the X-linked transcripts present in the array (88.5 %) were upregulated in females, and most of them (70 %), exhibited a fold change lower than 1.66, which suggests partial XCI. The distribution of genes that escape inactivation has been found to be not random along the chromosome, being clustered and map primarily to the distal portion of the X chromosome short arm (Xp) [66, 75], far from the *XIST* gene [76]. However, a relation between linear distribution

along the X-chromosome and fold change could not be established, which may be caused by the tridimensional structure of the chromosome.

The higher expression of X-linked genes in females is usually explained by a double allele expression. However, as males only contain the maternal inherited X-chromosome, a preferential paternal allele expression by an imprinting phenomenon may play a role in the sexual dimorphism [54]. The imprinting phenomenon was firstly discovered by the observation that mouse embryogenesis requires both the maternal and paternal genomes [77]. The imprint mark is set during gametogenesis [78], and result in the expression of the imprinted gene from only one of the two parental chromosomes [79], although partial imprinting mechanisms have been reported, specially during preimplantation development [80, 81]. Probably because XCI implies the random inactivation of one of the X-chromosomes, few X-linked imprinted genes have been reported [54]. Nevertheless, imprinting mechanisms have been proposed to evolve from mechanisms to defend the genome against transposable elements [82], and compared to autosomal chromosomes, the X chromosome has generated a disproportionately high number of functional retroposed genes in mammalian species [83]. In chapter 5, possible imprinting mechanisms were tested by analyzing the differences in expression levels of eight X-linked genes between male, female and parthenogenetic embryos. Five out of eight X-linked genes which displayed transcriptional sexual dimorphism were found to be expressed preferentially by the paternal X-chromosome. X-chromosome imprinting phenomena have clinical implications in human syndromes. Turner syndrome (XO) females differ in their cognitive and behavioural phenotypes according to the parental origin of their single X chromosome, being X^pO better socially adjusted that X^mO [84], although their visual memory was poorer [85]. Differences on superior temporal gyrus morphology have been also reported [86]. Similarly, X^pO female mice are more competent than X^mO [78]. Klinefelter syndrome (XXX) symptoms also depend on the origin of the supernumerary X-chromosome [87]. Interestingly, X-linked gene expression is higher in brain compared with other tissues [88]. In humans, brain expressed genes are in 5-fold excess on the X-chromosome compared with autosomal chromosomes [89], and X-chromosome contents a disproportionate number of genes involved in mental retardation syndromes [89, 90]. Four out of the five genes which were found to be preferentially expressed by the paternal allele were expressed in adult brain.

Imprinting mechanisms for X-linked genes may play a role in X-linked mental retardation syndromes, as it has been suggested for autism, which is four times more frequent in males than in females [91].

YZRSR2

The novel Y-linked transcript discovered in chapter 5 (*YZRSR2*) seems to have evolved from its X-linked homologue gene *ZRSR2*. For this gene pairs, the expression of the X-linked homologue (*ZRSR2*) in females is not sufficient to compensate for the male bias in *YZRSR2* gene expression. *YZRSR2* is located in the non-recombining portion of the Y-chromosome, which only contains genes responsible for large fitness effects such as those that determine sex or are required for male function [92]. Its function remains unclear, but it may be involved in splicing, as *ZRSR2* [93]. *ZRSR2* has an autosomal homologous in mouse (*ZRSR1*), which is a paternally expressed imprinted gene that is silenced during oogenesis [94]. Thus, it can be speculated that *YZRSR2* may have a role on sex determination, sex differentiation and/or sexual dimorphism by operating at the posttranscriptional level. Alternative splicing may originate transcriptional sexual dimorphism, as it has been reported for mouse liver [95]. Some of the factors involved in sex determination and differentiation have multiple spliced transcripts, such as *Dmrt1*, which has been proposed to govern sex differentiation in mouse [96]. *Dmrt1* is the only male development regulator conserved in the whole vertebrate phyla and sex-differentiation in *Drosophila* is governed by a splicing mechanism of the *Dmrt1* homologous gene *ddx* [97]. In some fish, bird, amphibians and monotremes, *Dmrt1* is sex chromosome-linked [98], but in eutherian mammals only one sex determination gene –*Sry*-, that is not present in monotremes and some rodents [99], has been described to be sex chromosome-linked. The finding of this splicing factor Y-chromosome linked in bovine, homologous to a paternally expressed autosomal gene silenced during oogenesis in mouse [94], together with the alternative splicing of *Dmrt1* found in mouse gonadal development [96], may suggest a role for splicing-based mechanisms in vertebrate sex differentiation.

DISCUSIÓN GENERAL

Evaluación de la supuesta selección espermática llevada a cabo por el ovocito

Existen dos grupos de mecanismos que pueden estar implicados en los sesgos de la proporción de sexos originados antes de la concepción. El primero implica una supuesta selección espermática llevada a cabo por el ovocito que puede alterar la igualdad de posibilidades de fecundación entre los espermatozoides X e Y. El segundo se basa en unas supuestas diferencias intrínsecas entre los espermatozoides X e Y que pueden perturbar la equidad de probabilidades de alcanzar el lugar de la fecundación entre los espermatozoides X e Y. En los capítulos 1 y 2 se probó la viabilidad de la primera hipótesis.

Los mecanismos de selección del sexo del espermatozoide presentes en el ovocito han sido propuestos para explicar la relación entre la proporción de sexos y tres características distintas del ovocito: el estado de maduración [1-3], la concentración de testosterona en el fluido folicular [4] y el lado del ovario de origen [5, 6]. La existencia de una supuesta selección del espermatozoide motivada por el estado de maduración del ovocito proporciona una explicación a la influencia del momento de inseminación en relación con la ovulación sobre la proporción de sexos descrita en varias especies, incluyendo a la bovina (discutido en la introducción). Se ha propuesto que el retraso en la inseminación permite a los ovocitos detenidos en metafase II procesar al esperma Y de forma más eficiente [1-3]. En el capítulo 1, se examinó el efecto del estado de maduración sobre la proporción de sexos, y se encontró que el tiempo de maduración afectó a la cinética de la primera división y a la tasa de división, como se ha discutido en el capítulo, pero no a la proporción de sexos.

La existencia de una supuesta selección espermática llevada a cabo por el ovocito tendría un gran impacto sobre la tecnología del semen sexado, ya que supone que las tasas de fertilidad seguirán siendo bajas a pesar del avance en las técnicas de separación espermática [7] (discutido en la introducción). En el capítulo 2, evaluamos una hipotética selección espermática llevada a cabo por el ovocito examinando las diferencias en fertilidad in vitro entre esperma sin sexar, sexado (X o Y) y sexado-recombinado (una mezcla de X e Y). Si el ovocito es capaz de seleccionar al esperma,

obtendríamos un descenso en la fertilidad en ambos grupos sexados (X o Y) y la fertilidad mejoraría en la mezcla de sexados (X e Y). En oposición a una supuesta selección espermática, se obtuvo un descenso en la fertilidad de los tres grupos fecundados con semen sexado comparados con el semen sin sexar.

Tres estudios realizados después de la publicación del capítulo 2 han confirmado la ausencia de un proceso selectivo de espermatozoides X o Y en el ovocito [8-10]. El primero empleó hibridación *in situ* para determinar el porcentaje de espermatozoides Y en el esperma epididimal de ratón y en los espermatozoides pegados a ovocitos de ratón madurados *in vivo*, y no encontró una desviación del teórico 1:1, concluyendo que los ovocitos de ratón no atraen de forma selectiva a una clase de espermatozoide [8]. Este descubrimiento concuerda con un estudio anterior que encontró el mismo número de espermatozoides bovinos X e Y entre aquellos que penetraban el ovoplasma bovino [11]. Los otros dos estudios abordan el efecto de la testosterona sobre los supuestos mecanismos del ovocito de selección del sexo [4]. El primero indica que el aumento de la concentración de testosterona en el medio de maduración no afecta a la proporción de sexos [9], mientras que el segundo no puede repetir los resultados del estudio de Grant *et al.* [10]. Un mecanismo de selección ovocitaria del esperma dependiente de testosterona proporcionaría una explicación fisiológica a la relación entre la dominancia materna y la proporción de sexo citada en varias especies (discutido en la introducción). Sin embargo, no se ha publicado una relación entre niveles altos de testosterona en suero, que están relacionados con la dominancia materna, y niveles altos de testosterona en folículos.

Se ha sugerido que el lado del ovario de origen de un ovocito puede influir en la probabilidad de que dicho ovocito sea fecundado por un espermatozoide X o Y. En esta linea, se ha afirmado que la proporción de sexos difiere entre los cuernos uterinos izquierdo y derecho en varias especies en las que la migración uterina es baja (discutido en la introducción). De ser cierta esta controvertida idea, la selección espermática llevada a cabo por el ovocito no sería el único mecanismo capaz de explicarlo: las opciones de que los espermatozoides X o Y alcancen el lugar de la fecundación pueden variar entre ambos lados del útero. De acuerdo con esta opción, un artículo publicado recientemente que observó diferentes proporciones de sexos en los cuernos uterinos bovinos derecho e izquierdo, no encontró variaciones en la

proporción de sexos ni en terneras ovariectomizadas unilateralmente ni en embriones producidos por FIV a partir de ovocitos procedentes de matadero con origen en ovarios derechos o izquierdos [5, 12].

Los resultados presentados en el capítulo 2 constituyen una clara evidencia en contra de la existencia de una selección de los espermatozoides sexados X o Y por parte del ovocito. Este mecanismo hipotético requiere de la presencia de proteínas específicas de sexo en la membrana del espermatozoide que permitirían que complejo cúmulo-ovocito los distinguiese, lo que es teóricamente posible, como se ha explicado en la introducción. Sin embargo, varios estudios han fracasado en la detección de esos antígenos específicos de sexo [13-15]. Además, este mecanismo podría implicar una reducción en la fertilidad, puesto que en la fecundación *in vivo*, un número limitado de espermatozoides alcanzan el lugar de la fecundación, donde un mecanismo de selección reduciría las posibilidades de fecundación. A diferencia de los ovocitos, que están presentes en un número reducido, cada inseminación aporta millones de espermatozoides, haciendo que sea más factible la selección espermática a lo largo del tracto genital femenino que un mecanismo de selección en el ovocito. Considerando lo anterior, estos resultados proponen a las diferencias intrínsecas entre los espermatozoides X e Y, que pueden alterar la equidad de posibilidades de alcanzar el lugar de fecundación, como responsables de los mecanismos preconceptuales de selección del sexo. Estos mecanismos independientes del ovocito podrían explicar los posibles efectos preconceptuales de distintos parámetros de la hembra sobre la proporción de sexos, como el momento de inseminación, los niveles de testosterona o la condición corporal.

Análisis de la baja fertilidad obtenida con semen sexado

Como se ha descrito en la introducción, el uso de semen sexado se asocia frecuentemente a un descenso en las tasas de fertilidad en condiciones *in vivo* e *in vitro*, impidiendo su uso en sistemas de producción animal convencionales. Esta reducción de la fertilidad puede deberse a diferencias en la calidad del esperma (daño espermático durante el procedimiento de separación y bajas dosis de espermatozoides) o a un supuesto mecanismo de selección del ovocito. Como ya se ha mencionado, los resultados del capítulo 2 contradicen a la segunda posibilidad. Además, a pesar de

usar la misma concentración de espermatozoides vivos seleccionados por Percoll, en los capítulos 2 y 3 se observó una reducción en la fertilidad al usar esperma sexado. Como se ha revisado en el capítulo 3, el efecto deletéreo causado por el procedimiento de separación en los espermatozoides es el responsable de las bajas tasas de fertilidad.

Los datos de granja disponibles muestran un aumento en el porcentaje de vacas que repiten celo en intervalos interestrales normales tras la inseminación con semen sexado [16]. Esta situación se puede deber a una reducción en la capacidad fecundante del semen sexado o a un incremento en la mortalidad embrionaria anterior al reconocimiento materno. Para distinguir entre ambas posibilidades, en nuestro estudio *in vitro* se anotó la tasa de división en distintos momentos de observación y el porcentaje de blastocistos. Se detectó una reducción en ambos parámetros en los grupos fecundados con semen sexado, pero la proporción blastocisto/embrión de división temprana (antes de 33 hpi) fue similar, sugiriendo que no había un aumento de la mortalidad embrionaria y que la reducción de fertilidad se debía a una reducción en la capacidad fecundante. Además se obtuvo un retraso en el momento de la primera división al usar esperma sexado, que puede estar asociado a la reducción en la motilidad descrita en espermatozoides sexados criopreservados [17, 18]. Este retraso también puede ser causado por un aumento en el tiempo de procesamiento del espermatozoide por el ovocito tras la fecundación con semen sexado, ya que en ratones, la ICSI con esperma con ADN fragmentado (30 %) causa un retraso de 2 h en la demetilación activa del pronucleo masculino [19]. Sin embargo, se ha estimado que el daño potencial en el ADN del esperma sexado, incluyendo la tinción y la iluminación con láser, es un 3,2 % mayor que el del esperma sin sexar [20], lo que quizás no suponga consequencia alguna. Es interesante destacar que la alteración de la evolución de la primera división difirió de forma significativa entre semen sexado procedente de distintos toros, coincidiendo con la diferencias en fertilidad entre toros en FIV [21-23] o IA [24]. Este resultado sugiere que la susceptibilidad del esperma al daño espermático causado por el procedimiento de separación puede variar entre toros, y la FIV puede ser una valiosa herramienta para analizar estas diferencias en susceptibilidad y, así, permitir la selección del semental para obtener altas tasas de fertilidad en IA o realizar mejoras en el procedimiento de sexaje.

Los espermatozoides dañados pueden ser capaces de fecundar ovocitos dando lugar a embriones de baja calidad [19], que pueden implantar y ser gestados dando lugar a efectos negativos a largo plazo en la descendencia [25], hecho que limitaría seriamente el uso de la tecnología de semen sexado en el ganado vacuno y en humanos. En el capítulo 3, se analizó un supuesto efecto negativo del uso de semen sexado sobre 2 parámetros de calidad embrionaria (velocidad de desarrollo y expresión génica). El uso de semen sexado no afectó ni al momento de aparición del blastocisto ni a la abundancia relativa de transcritos de genes relacionados con apoptosis, detoxificación de radicales de oxígeno inducidos por ultravioleta, reconocimiento de gestación, formación de placenta y metilación de ADN. Estos resultados, en combinación con la normalidad de la descendencia obtenida con semen sexado [26], sugieren que el sexaje de esperma no afecta a la calidad embrionaria.

Dimorfismo sexual en velocidad de desarrollo y supervivencia en el medio de cultivo

Para determinar el posible dimorfismo sexual en velocidad de desarrollo y supervivencia en el medio de cultivo se emplearon dos aproximaciones diferentes. En primer lugar, en el capítulo 1, se sexaron embriones producidos con semen sin sexar en los estadios de 2 células o blastocisto en distintos momentos de aparición. De existir un dimorfismo sexual en velocidad de desarrollo, se esperarían cambios en la proporción de sexos observada en distintos momentos de aparición, mientras que si sucede una pérdida selectiva de embriones de un sexo determinado, se deberían observar distintas proporciones de sexos entre los estadios de 2 células y blastocisto. En segundo lugar, en el capítulo 3, se anotó la evolución de la primera división y la aparición de blastocistos en grupos de embriones producidos por FIV con semen sexado X o Y (aproximadamente 90 % de exactitud en el sexaje). En este caso, se deberían detectar diferencias en la evolución de la primera división y la aparición de blastocistos al usar semen sexado X o Y del mismo toro si existieran diferencias entre sexos en velocidad de desarrollo; y se deberían encontrar diferencias en la proporción blastocisto/embrión dividido entre grupos fecundados con semen sexado X o Y del mismo toro en el caso de que las tasas de supervivencia fueran específicas de sexo. No se encontraron diferencias entre sexos en velocidad de desarrollo o supervivencia

en ambas aproximaciones. Como se ha revisado en la introducción, estas diferencias entre sexos pueden aparecer en condiciones adversas.

Tras el análisis de los datos de los capítulos 1-3, se detectó un aumento de la proporción de sexos después de la FIV. En el capítulo 1, el porcentaje de machos a las 48 hpi fue de 55,9 % y 59,1 % en los grupos madurados durante 24 y 16 horas, respectivamente. De forma similar, en el capítulo 2 el porcentaje de machos en los embriones de 2 o 4 células sexados estuvo en torno al 57 % en los grupos fecundados con semen sin sexar o con la mezcla de sexados. Por último, se obtuvo una tasa de división mayor al usar semen sexado Y en comparación con el X en los tres toros analizados. En conjunto, estos resultados sugieren que la capacidad fecundante de los espermatozoides Y en FIV puede ser ligeramente superior que la de los X. Se ha descrito que la proporción de sexos secundaria en bovinos está aumentada, con un 52,9 % de machos sobre 500000 partos de ganado lechero [27]. De forma similar, en humanos, aunque el porcentaje de espermatozoides Y es cercano al 50,3 %, la proporción de sexos de los nacidos vivos está en torno al 51,3 % en varios países [28]. Varios estudios estadísticos han confirmado que la proporción de sexos humana es aproximadamente 106:100, con variaciones entre distintos países (revisado en [29]). Además, tiene lugar una mayor pérdida de fetos macho que de hembras [30, 31]. A consecuencia de lo anterior, se ha propuesto que la proporción de sexos primaria puede apartarse en gran medida de la igualdad esperada teóricamente en humanos [29]. De acuerdo con esta hipótesis, se ha calculado que la proporción de sexos en embriones humanos es 164:100 y que disminuye a 111:100 en el estadio fetal en la población finlandesa [32].

Dimorfismo sexual preimplantacional epigenético y transcripcional

El estudio del dimorfismo sexual preimplantacional puede ayudar al entendimiento de la pérdida selectiva de embriones de un sexo determinado que acontece en condiciones adversas y puede ser responsable de las variaciones de la proporción de sexos que ocurren después de la concepción y están ligadas a una reducción en la fertilidad. Las diferencias fisiológicas observadas bajo condiciones subóptimas *in vitro* –relativas a la velocidad de desarrollo, la supervivencia a la vitrificación y al número de células (revisadas en la introducción)- deben tener su

origen a nivel molecular. Sin embargo, a este nivel sólo se han publicado ciertas observaciones basadas en la transcripción de algunos genes específicos y en el metabolismo de la glucosa.

La epigenética es la base de las diferencias transcripcionales que modulan la traducción dando lugar a las diferencias funcionales. En el capítulo 4, se encontraron diferencias entre sexos en la abundancia relativa de ARNm de genes relacionados con la regulación epigenética de la transcripción. En concreto, las dos *de novo* ADN-metiltransferasas se expresaron a un mayor nivel en los embriones macho, sugiriendo que el genoma de los embriones macho puede estar más metilado, y por ello más silenciado [33] en comparación con las hembras. El descubrimiento de un mayor nivel de metilación en una de las cinco secuencias analizadas, confirmó esta hipótesis y sugiere que las diferencias entre sexos en metilación no afectan a todo el genoma, sino que son específicas de secuencia o región genómica. Por otra parte, la transcripción de dos enzimas relacionadas con la metilación de histonas y por tanto con silenciamiento genómico [33], fue superior en embriones macho. Además, el análisis de ontología génica del capítulo 5 sugiere un mayor nivel de transcripción global en los embriones hembra, de acuerdo con los resultados del capítulo 4. Después de la publicación del capítulo 4, se han aportado nuevas evidencias de dimorfismo sexual en el estatus de metilación en bovinos. Gebert *et al.* han observado que el nivel de metilación de una región metilada de forma diferencial (RMD) del gen *IGF2* fue menor en los blastocistos hembra que en los macho [34]. Se han encontrado niveles bajos de metilación en CGE y CGP hembra en comparación con las células macho, aunque estas diferencias pueden deberse al efecto demetilante del anillo genital femenino (revisado en [34]). Sin embargo, se ha visto en células troncales embrionarias de ratón, que derivan directamente del blastocisto, que las líneas XX están hipometiladas con respecto a las lineas XY [35], y se ha propuesto que el cromosoma X puede codificar un locus modificador cuyo producto inhibe a las *de novo* ADN-metiltransferasas [35]. Teniendo en cuenta que en el capítulo 5 se observó que en el estadio de blastocisto la inactivación del cromosoma X está lejos de su terminación, es probable que este locus se exprese a mayor nivel en hembras, que de este modo mostrarían bajos niveles de estas enzimas. El dimorfismo sexual a nivel epigenético también se ha descrito en ratones neonatos, con mayores niveles de acetilación y metilación de histonas en machos [36]. Llama la atención que el nivel de acetilación

de histonas pareció deberse a la exposición a testosterona, mientras que el mayor nivel de metilación de histonas no pudo conseguirse en hembras expuestas a testosterona [36]. Estas diferencias específicas de sexo en niveles de metilación pueden dar lugar a efectos a largo plazo, como se discutirá más adelante.

Se observó que los telómeros son más cortos en los blastocistos macho (capítulo 4). El alargamiento telomérico durante el desarrollo temprano es especialmente importante, porque las reservas de telómeros en recién nacidos se establecen en la transición de mórula a blastocisto [37] y, por tanto, las diferencias en longitud telomérica en el estadio de blastocisto pueden tener consecuencias a largo plazo. En este sentido, se ha descrito que, en el momento del nacimiento, los telómeros del macho son más cortos en ratones y ratas [38, 39], y en el brazo Xq en humanos [40]. El establecimiento de una relación entre esta observación y la menor esperanza de vida del macho en distintas especies, incluída la humana [41] puede ser demasiado especulativa, ya que hay muchos factores que influyen sobre la mortalidad específica de sexo. El mecanismo molecular responsable de esta diferencia no ha sido dilucidado, aunque se ha identificado un locus ligado al cromosoma X que ejerce un gran efecto sobre la longitud telomérica [42] y las *de novo* ADN-metiltransferasas son reguladores negativos de la longitud telomérica [43].

Las mitocondrias son los orgánulos más abundantes en el ovocito y embrión temprano de los mamíferos [44] y se ha demostrado que tienen un papel crítico en el desarrollo preimplantacional [45-47], ya que son esenciales para la glucolisis oxidativa, cuya importancia aumenta en la transición de mórula a blastocisto (revisado en la introducción y en [48]). Durante el desarrollo preimplantacional, cada mitocondria contiene una única copia de ADN mitocondrial, permitiendo su cuantificación mediante qPCR [49]. Se ha observado que el ADN mitocondrial se replica durante el desarrollo preimplantacional en ratones y bovinos [50, 51], abriendo una vía a la existencia de diferencias entre sexos. Además, se ha indicado que el ADN mitocondrial puede estar implicado en el dimorfismo sexual [52]. En el capítulo 4, se observó un mayor contenido en ADN mitocondrial en embriones macho. En la misma tendencia, en el capítulo 5, el análisis de ontología génica mostró que entre los componentes celulares, las mitocondrias y sus membranas internas estaban sobrerrepresentados entre los genes sobreexpresados en machos. El dimorfismo

sexual en el contenido de ADN mitocondrial puede explicar las diferencias en metabolismo de glucosa y susceptibilidad a estrés oxidativo entre embriones macho y hembra [53].

Como ya se ha revisado en la introducción, se han descrito diferencias entre sexos en la expresión de genes ligados a cromosomas sexuales y autosómicos. Sin embargo, salvo en el caso de un estudio de *microarray* llevado a cabo en el modelo murino [54], todas las observaciones se han basado en un número limitado de genes. En los capítulos 3 y 4, se describió que la expresión de ciertos genes (*DNMT3A*, *DNMT3B*, *HMT1*, *ILF3*, *GSTM3* and *PGRMC1*) exhibía un dimorfismo sexual previamente desconocido. Los análisis a gran escala de las diferencias transcripcionales son necesario para la evaluación de la extensión del dimorfismo sexual preimplantacional en la expresión génica y para validar los resultados previos observados en estudios que analizaron un escaso número de genes. En el capítulo 5, se observó que en la ausencia de diferencias hormonales, los cromosomas sexuales imponen una extensa regulación transcripcional sobre los genes autosómicos, afectando a casi una tercera parte (2921) de los transcritos detectados. El *array* se llevó a cabo en embriones producidos *in vitro* con semen sexado, pero la validación se realizó sobre embriones producidos *in vitro* e *in vivo* con semen sin sexar. Estos resultados conllevan importantes repercusiones para los estudios de biología del desarrollo y regulación genómica. Los embriones preimplantacionales constituyen un modelo ideal para el estudio de la expresión génica dependiente del sexo sin influencias hormonales. Nuestros resultados concuerdan con los fenómenos de dimorfismo sexual en la expresión génica encontrados en células adultas (discutidos en el capítulo 5), y sugieren que gran parte de la expresión génica dependiente del sexo puede deberse directamente a los cromosomas sexuales en vez de a niveles hormonales. El extenso dimorfismo sexual preimplantacional al nivel transcripcional tiene repercusiones sobre la mortalidad embrionaria específica de sexo, la inactivación del cromosoma X y efectos a largo plazo, como será discutido posteriormente. La ontología génica estuvo de acuerdo con las observaciones previas, como las diferencias en el estatus epigenético y en el ADN mitocondrial. Además, los genes relacionados con el metabolismo proteico estuvieron sobrerrepresentados entre los genes sobreexpresados en machos, lo que puede tener un papel en el dimorfismo sexual en el recambio aminoacídico que se ha descrito recientemente en blastocistos

bovinos [55]. Otra repercusión importante de los resultados aquí descritos es que al realizar análisis en embriones individuales, los datos de los machos y las hembras deberían analizarse de forma separada, ya que las diferencias transcripcionales encontradas pueden deberse al dimorfismo sexual en vez de ser causadas por el tratamiento. Se puede decir lo mismo de la comparación de embriones producidos mediante transferencia nuclear de una línea celular, que son por tanto XX o XY, con un conjunto de embriones XX y XY producidos por FIV u obtenidos *in vivo*.

La existencia de condiciones ambientales subóptimas durante el desarrollo preimplantacional puede dar lugar a efectos a largo plazo en la descendencia causadas por alteraciones en la reprogramación epigenética [25]. En muchos casos, se ha descrito que los efectos a largo plazo son específicos del sexo, en concordancia con las diferencias epigenéticas observadas en los capítulos 4 y 5. Probablemente, el efecto a largo plazo más estudiado es el síndrome del ternero gigante, causado por condiciones de cultivo *in vitro* subóptimas y caracterizado por un crecimiento desproporcionado del feto acompañado de una reducción en su viabilidad [56]. La presencia de suero en el medio de cultivo de los embriones de ratón produce unos efectos a largo plazo similares al síndrome del ternero gigante, que difieren entre embriones macho y hembra; en particular se encontró un aumento del peso corporal sólo en hembras y varias anomalías del comportamiento también dependieron del sexo [57]. En el mismo animal, pero usando otro modelo (presencia o ausencia de un factor de crecimiento en el medio de cultivo de los embriones), otro estudio asoció las condiciones subóptimas de cultivo *in vitro* con un aumento del peso corporal y un descenso del tamaño relativo del cerebro en machos, pero no en hembras [58]. En humanos, el síndrome de Beckwith-Wiedemann es un desorden epigenético con origen en el periodo preimplantacional que se considera equivalente al síndrome del ternero gigante, ya que se caracteriza por alteraciones similares y se ha relacionado con patrones de metilación de RMDs de genes implicados en la señalización de IGF2 (como la hipometilación de *KvDMR1*) [59]. Es remarcable el hecho de que el sindrome de Beckwith-Wiedemann es relativamente frecuente en gemelos monocigóticos, y en la mayoría de los casos, los gemelos afectados son hembra [60, 61], en coincidencia con el mayor estatus de metilación encontrado en los embriones macho en los capítulos 4 y 5 y en [34]. Además, en oveja, la administración de una dieta materna deficiente en metilos durante el periodo periconcepcional da lugar a

efectos a largo plazo en la descendencia mediante la alteración de los niveles de metilación de ciertos *loci* de un modo específico del sexo: más de la mitad de los *loci* afectados fueron específicos de machos, correspondiéndose con una mayor relevancia clínica del efecto fenotípico en ese sexo [62]. Las diferencias en el metabolismo proteico sugeridas en el capítulo 5 y en [55], pueden dar lugar a efectos a largo plazo. En ratas, el empleo de una dieta materna baja en proteínas durante el periodo preimplantacional produjo efectos a largo plazo específicos de género, como un descenso en el peso al nacimiento en la descendencia hembra y un aumento de la presión sistólica y proporciones órgano/cuerpo anormales en la descendencia macho [63].

La inactivación del cromosoma X en blastocistos bovinos

Para compensar la pérdida gradual de genes en el cromosoma Y a lo largo de la evolución, los mamíferos desarrollaron el mecanismo de inactivación del cromosoma X (ICX). En mamíferos euterios, la ICX tiene lugar en el cromosoma X paterno o materno de forma aleatoria durante la embriogénesis temprana. Algunos estudios consideran que la ICX está virtualmente completa en distintos tejidos adultos. El grado de realización de la ICX varía entre estudios en función del tejido [64], desde un 5,4 % de genes del cromosoma X con una mayor expresión en hembras en lineas celulares linfoblastoides humanas [65] a un 15-25 % en fibroblastos o células híbridas [66]. La ICX fue propuesta por primera vez en 1961 para explicar los fenotipos mosaico observados en ratonas heterocigotas a mutaciones ligadas al sexo en genes del color de la capa [67] y fue confirmada más tarde en mujeres heterocigotas a distintos genes del cromosoma X, como *G6PD*, *HPRT* y *PGK* [68-71]. En los embriones de ratón, el cromosoma X paterno está completamente activo tras la activación del genoma embrionario [72], después sufre una inactivación marcada parentalmente a partir de los estadios de división, y en las células de la masa celular interna del blastocisto expandido o eclosionado sucede una inversión del estado inactivo, con una pérdida de las marcas epigenéticas como la modificación de histonas o las proteínas *polycomb*, que dan lugar a la inactivación aleatoria del cromosoma X propia del embrión [73]. Sin embargo, se conoce poco de la ICX durante el periodo preimplantacional en otras especies, y puede haber grandes variaciones entre mamíferos [74]. En el capítulo 5, observamos que en el blastocisto bovino, la ICX

está lejos de su terminación. De acuerdo con la presencia de ambos cromosomas parcialmente activos, se vió que la mayoría de los transcritos ligados al cromosoma X presentes en el *array* (88,5 %) estaban sobreexpresados en las hembras, y la mayoría (70 %) mostró un nivel de cambio de expresión inferior a 1,66, sugiriendo una ICX parcial. Se ha observado que la distribución de los genes que escapan a la inactivación a lo largo del cromosoma no es aleatoria, sino agrupada y localizada principalmente en la porción distal del brazo corto (Xp) del cromosoma X [66, 75], lejos del gen *XIST* [76]. Sin embargo, no se pudo establecer una relación entre la distribución lineal a lo largo del cromosoma X y el nivel de cambio de expresión, posiblemente debido a la estructura tridimensional del cromosoma.

El mayor nivel de expresión de los genes del cromosoma X en hembras se suele explicar mediante la expresión de ambos alelos. Sin embargo, dado que los machos sólo contienen el cromosoma X heredado de la madre, una expresión preferente por parte del alelo paterno mediante un fenómeno de impronta genómica parental puede tener un papel en el dimorfismo sexual [54]. El fenómeno de la impronta genómica parental se descubrió por primera vez gracias a la observación de que los genomas paterno y materno eran necesarios para la embriogénesis del ratón [77]. La marca de esta impronta se establece durante la gametogénesis [78], y desemboca en que la expresión del gen marcado parentalmente sólo se produce por parte de uno de los dos cromosomas parentales [79], aunque se han descrito mecanismos de impronta genómica parental parciales, especialmente durante el desarrollo preimplantacional [80, 81]. Se han descrito pocos genes marcados parentalmente [54], probablemente debido a que la ICX implica la inactivación aleatoria de uno de los dos cromosomas X. Sin embargo, se ha propuesto que los mecanismos de impronta genómica parental han evolucionado a partir de mecanismos de defensa del genoma frente a elementos transponibles [82] y, en comparación con los cromosomas autosómicos, el cromosoma X ha generado un número desproporcionadamente alto de genes funcionales retrotranspuestos en los mamíferos [83]. En el capítulo 5, se determinaron los posibles mecanismos de impronta genómica parental analizando las diferencias en el nivel de expresión de ocho genes del cromosoma X entre embriones macho, hembra y partenotes. Cinco de los ocho genes del cromosoma X que mostraron un elevado dimorfismo sexual transcripcional, fueron expresados de forma preferente por el cromosoma X paterno. Los fenómenos

de impronta genómica parental del cromosoma X tienen implicaciones clínicas en síndromes humanos. Las hembras con síndrome de Turner (XO) difieren en sus fenotipos cognitivos y de comportamiendo según el origen parental de su único cromosoma X, mejorando la adaptación social en X^pO comparado con X^mO [84], aunque su memoria visual fue menor [85]. También se han descrito diferencias en la morfología del giro temporal superior [86]. De forma similar, las ratonas X^pO son más competentes que las X^mO [78]. Los síntomas del síndrome de Klinefelter (XXX) también dependen del origen del cromosoma X supernumerario [87]. Es destacable que la expresión de genes del cromosoma X es más alta en cerebro en comparación con otros tejidos [88]. En humanos, el porcentaje de genes expresados en el cerebro presentes en el cromosoma X es 5 veces superior a la de los cromosomas autosómicos [89] y el cromosoma X contiene un número desproporcionado de genes implicados en síndromes de retraso mental [89, 90]. Cuatro de los cinco genes expresados de forma preferente por el alelo paterno son expresados en el cerebro adulto. Los mecanismos de impronta genómica parental de los genes del cromosoma X pueden tener un papel en los síndromes de retraso mental ligados al cromosoma X, como se ha propuesto en el caso del autismo, que es cuatro veces más frecuente en machos que en hembras [91].

YZRSR2

El nuevo transcrito ligado al cromosoma Y descubierto en el capítulo 5 (*YZRSR2*) parece haber evolucionado de su gen homólogo en el cromosoma X *ZRSR2*. Para esta pareja de genes, la expresión del homólogo del cromosoma X (*ZRSR2*) no es capaz de compensar el sesgo en la expresion de *YZRSR2* en machos. *YZRSR2* se localiza en la región no recombinante del cromosoma Y, que sólo contiene genes con grandes efectos adaptativos como aquellos que determinan el sexo o son necesarios para la función del macho [92]. Su función es desconocida, pero puede estar implicado en el ensamblado de transcritos, como *ZRSR2* [93]. *ZRSR2* tiene un homólogo autosómico en ratones *(ZRSR1*), que es un gen marcado parentalmente de expresión paterna que se silencia durante la ovogénesis [94]. De este modo, se puede especular con la posibilidad de que *YZRSR2* tenga un papel en la determinación sexual, la diferenciación sexual y/o el dimorfismo sexual mediante acciones a nivel posttranscripcional. El ensamblado alternativo de transcritos puede dar lugar a un

dimorfismo sexual transcripcional, como se ha descrito en hígado de ratón [95]. Algunos de los factores implicados en la determinación del sexo y en la diferenciación sexual tienen múltiples transcritos originados por el ensamblado alternativo, como *Dmrt1*, que se ha propuesto como candidato para el control de la diferenciación sexual en ratones [96]. *Dmrt1* es el único regulador del desarrollo del macho conservado en todo el filo vertebrado y la diferenciación sexual en *Drosophila* está gobernada por un mecanismo de ensamblado alternativo de transcritos de *ddx*, el gen homólogo a *Dmrt1* [97]. En algunos peces, aves, anfibios y monotremas, *Dmrt1* está ligado a cromosomas sexuales [98], pero en mamíferos euterios sólo se ha descrito un gen de determinación del sexo ligado a cromosomas sexuales –*Sry*-, que no está presente en monotremas y algunos roedores [99]. El descubrimiento de este factor de ensamblado de transcritos ligado al cromosoma Y en bovinos, homólogo a un gen autosómico expresado por el alelo paterno y silenciado durante la ovogénesis en ratones [94], junto con el ensamblado alternativo de transcritos de *Dmrt1* encontrado en el desarrollo de la gónada de los ratones [96], puede indicar un papel de los mecanismos basados en el ensamblado de transcritos en la diferenciación sexual de los vertebrados.

Bibliography/Bibliografía

1. Dominko T, First NL. Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. Theriogenology 1997; 47: 1041-1050.
2. Gutierrez-Adan A, Perez G, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. Zygote 1999; 7: 37-43.
3. Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, Nagai T. Effect of maturation culture period of oocytes on the sex ratio of in vitro fertilized bovine embryos. Journal of Reproduction and Development 2006; 52: 123-127.
4. Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. Biol Reprod 2008; 78: 812-815.
5. Hylan D, Giraldo AM, Carter JA, Gentry GT, Jr., Bondioli KR, Godke RA. Sex ratio of bovine embryos and calves originating from the left and right ovaries. Biol Reprod 2009; 81: 933-938.
6. Clark MM, Ham M, Galef BG, Jr. Differences in the sex ratios of offspring originating in the right and left ovaries of Mongolian gerbils (Meriones unguiculatus). J Reprod Fertil 1994; 101: 393-396.
7. Grant V, Chamley L. Sex-sorted sperm and fertility: an alternative view. Biology of Reproduction 2007; 76: 184-188.
8. Mao J, Rosenfeld CS. Usage of X- and Y-chromosome fluorescent in situ hybridization to determine whether the murine oocytes selectively attract one class of spermatozoa over another. Mol Reprod Dev 2009; 76: 320.
9. Diez C, Bermejo-Alvarez P, Trigal B, Caamano JN, Munoz M, Molina I, Gutierrez-Adan A, Carrocera S, Martin D, Gomez E. Changes in testosterone or temperature during the in vitro oocyte culture do not alter the sex ratio of bovine embryos. J Exp Zool A Ecol Genet Physiol 2009; 311: 448-452.
10. García-Herreros M, Bermejo-Alvarez P, Rizos D, Gutiérrez-Adán A, Fahey AG, Lonergan P. Intrafollicular testosterone concentration and sex ratio in individually cultured bovine embryos. Reproduction Fertility and Development 2010; In press.
11. Zuccotti M, Sebastiano V, Garagna S, Redi CA. Experimental demonstration that mammalian oocytes are not selective towards X- or Y-bearing sperm. Mol Reprod Dev 2005; 71: 245-246.
12. Hylan DA. In utero and in vitro sex ratio of bovine embryos and calves originating from the left and right ovaries. Thesis, Luisiana Tech University 2007.
13. Howes EA, Miller NG, Dolby C, Hutchings A, Butcher GW, Jones R. A search for sex-specific antigens on bovine spermatozoa using immunological and biochemical techniques to compare the protein profiles of X and Y chromosome-bearing sperm populations separated by fluorescence-activated cell sorting. J Reprod Fertil 1997; 110: 195-204.
14. Sills ES, Kirman I, Colombero LT, Hariprashad J, Rosenwaks Z, Palermo GD. H-Y antigen expression patterns in human X- and Y-chromosome-bearing spermatozoa. Am J Reprod Immunol 1998; 40: 43-47.
15. Hendriksen PJ. Do X and Y spermatozoa differ in proteins? Theriogenology 1999; 52: 1295-1307.
16. DeJarnette JM, Nebel RL, Marshall CE. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. Theriogenology 2009; 71: 49-58.
17. Schenk JL, Suh TK, Cran DG, Seidel GE, Jr. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 1999; 52: 1375-1391.
18. Hollinshead FK, Gillan L, O'Brien JK, Evans G, Maxwell WM. In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. Reprod Fertil Dev 2003; 15: 351-359.
19. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR, Gutierrez-Adan A. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod 2008; 78: 761-772.
20. Garner DL. Flow cytometric sexing of mammalian sperm. Theriogenology 2006; 65: 943-957.
21. Palma GA, Olivier NS, Neumuller C, Sinowatz F. Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts. Anat Histol Embryol 2008; 37: 67-73.

22. Blondin P, Beaulieu M, Fournier V, Morin N, Crawford L, Madan P, King WA. Analysis of bovine sexed sperm for IVF from sorting to the embryo. Theriogenology 2008.
23. Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, Moreno JF, Dinnyes A, Ji W, Tian XC, Yang X, Du F. Developmental potential of vitrified holstein cattle embryos fertilized in vitro with sex-sorted sperm. J Dairy Sci 2006; 89: 2510-2518.
24. Hayakawa H, Hirai T, Takimoto A, Ideta A, Aoyagi Y. Superovulation and embryo transfer in Holstein cattle using sexed sperm. Theriogenology 2008.
25. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 2004; 101: 5880-5885.
26. Tubman LM, Brink Z, Suh TK, Seidel GE, Jr. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. J Anim Sci 2004; 82: 1029-1036.
27. Powell RL, Norman HD, Dickinson FN. Sire differences in sex ratio of progeny. J. Dairy Sci. 1975; 58: 1723-1726.
28. Graffelman J, Hoekstra R. A statistical analysis of the effect of warfare on the human secondary sex ratio. Human Biology 2000; 72: 433-445.
29. Pergament E, Toydemir PB, Fiddler M. Sex ratio: a biological perspective of 'Sex and the City'. Reprod Biomed Online 2002; 5: 43-46.
30. Vatten LJ, Skjaerven R. Offspring sex and pregnancy outcome by length of gestation. Early Hum Dev 2004; 76: 47-54.
31. McMillen MM. Differential mortality by sex in fetal and neonatal deaths. Science 1979; 204: 89-91.
32. Kellokumpu-Lehtinen P, Pelliniemi LJ. Sex ratio of human conceptuses. Obstet Gynecol 1984; 64: 220-222.
33. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33 Suppl: 245-254.
34. Gebert C, Wrenzycki C, Herrmann D, Groger D, Thiel J, Reinhardt R, Lehrach H, Hajkova P, Lucas-Hahn A, Carnwath JW, Niemann H. DNA methylation in the IGF2 intragenic DMR is re-established in a sex-specific manner in bovine blastocysts after somatic cloning. Genomics 2009; 94: 63-69.
35. Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R, Brockdorff N. Global hypomethylation of the genome in XX embryonic stem cells. Nat Genet 2005; 37: 1274-1279.
36. Tsai HW, Grant PA, Rissman EF. Sex differences in histone modifications in the neonatal mouse brain. Epigenetics 2009; 4: 47-53.
37. Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL. Telomere length is reset during early mammalian embryogenesis. Proc Natl Acad Sci U S A 2004; 101: 8034-8038.
38. Cherif H, Tarry JL, Ozanne SE, Hales CN. Ageing and telomeres: a study into organ- and gender-specific telomere shortening. Nucleic Acids Res 2003; 31: 1576-1583.
39. Coviello-McLaughlin GM, Prowse KR. Telomere length regulation during postnatal development and ageing in Mus spretus. Nucleic Acids Res 1997; 25: 3051-3058.
40. Perner S, Bruderlein S, Hasel C, Waibel I, Holdenried A, Ciloglu N, Chopurian H, Nielsen KV, Plesch A, Hogel J, Moller P. Quantifying telomere lengths of human individual chromosome arms by centromere-calibrated fluorescence in situ hybridization and digital imaging. Am J Pathol 2003; 163: 1751-1756.
41. Eskes T, Haanen C. Why do women live longer than men? Eur J Obstet Gynecol Reprod Biol 2007; 133: 126-133.
42. Callicott RJ, Womack JE. Real-time PCR assay for measurement of mouse telomeres. Comp Med 2006; 56: 17-22.
43. Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 2006; 8: 416-424.
44. Calarco PG. Polarization of mitochondria in the unfertilized mouse oocyte. Dev Genet 1995; 16: 36-43.
45. Tamassia M, Nuttinck F, May-Panloup P, Reynier P, Heyman Y, Charpigny G, Stojkovic M, Hiendleder S, Renard JP, Chastant-Maillard S. In vitro embryo production efficiency in cattle and its association with oocyte adenosine triphosphate content, quantity of mitochondrial DNA, and mitochondrial DNA haplogroup. Biol Reprod 2004; 71: 697-704.

46. Reynier P, May-Panloup P, Chretien MF, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 2001; 7: 425-429.
47. Bruggerhoff K, Zakhartchenko V, Wenigerkind H, Reichenbach HD, Prelle K, Schernthaner W, Alberio R, Kuchenhoff H, Stojkovic M, Brem G, Hiendleder S, Wolf E. Bovine somatic cell nuclear transfer using recipient oocytes recovered by ovum pick-up: effect of maternal lineage of oocyte donors. Biol Reprod 2002; 66: 367-373.
48. Bermejo-Alvarez P, Lonergan P, Rizos D, Gutiérrez-Adán A. Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolisis. Reproductive Biomedicine Online 2010; in press.
49. Cummins JM. The role of maternal mitochondria during oogenesis, fertilization and embryogenesis. Reprod Biomed Online 2002; 4: 176-182.
50. May-Panloup P, Vignon X, Chretien MF, Heyman Y, Tamassia M, Malthiery Y, Reynier P. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. Reprod Biol Endocrinol 2005; 3: 65.
51. McConnell JM, Petrie L. Mitochondrial DNA turnover occurs during preimplantation development and can be modulated by environmental factors. Reprod Biomed Online 2004; 9: 418-424.
52. Mittwoch U. The elusive action of sex-determining genes: mitochondria to the rescue? J Theor Biol 2004; 228: 359-365.
53. Perez-Crespo M, Ramirez MA, Fernandez-Gonzalez R, Rizos D, Lonergan P, Pintado B, Gutierrez-Adan A. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol Reprod Dev 2005; 72: 502-510.
54. Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, Nakanishi T, Ikawa M, Hamada H, Abe K, Okabe M. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. Curr Biol 2006; 16: 166-172.
55. Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Mol Reprod Dev 2010; 77: 285-296.
56. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27: 153-154.
57. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez M, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proceedings of Natural Academy of Science USA 2004; 101: 5880-5885.
58. Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. Endocrinology 2005; 146: 2142-2153.
59. Weksberg R, Shuman C, Smith AC. Beckwith-Wiedemann syndrome. Am J Med Genet C Semin Med Genet 2005; 137C: 12-23.
60. Lubinsky MS, Hall JG. Genomic imprinting, monozygous twinning, and X inactivation. Lancet 1991; 337: 1288.
61. Weksberg R, Shuman C, Caluseriu O, Smith AC, Fei YL, Nishikawa J, Stockley TL, Best L, Chitayat D, Olney A, Ives E, Schneider A, Bestor TH, Li M, Sadowski P, Squire J. Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. Hum Mol Genet 2002; 11: 1317-1325.
62. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG, Young LE. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 2007; 104: 19351-19356.
63. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 2000; 127: 4195-4202.
64. Talebizadeh Z, Simon SD, Butler MG. X chromosome gene expression in human tissues: male and female comparisons. Genomics 2006; 88: 675-681.

65. Johnston CM, Lovell FL, Leongamornlert DA, Stranger BE, Dermitzakis ET, Ross MT. Large-scale population study of human cell lines indicates that dosage compensation is virtually complete. PLoS Genet 2008; 4: e9.
66. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature 2005; 434: 400-404.
67. Lyon MF. Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature 1961; 190: 372-373.
68. Deys BF, Grzeschick KH, Grzeschick A, Jaffe ER, Siniscalco M. Human phosphoglycerate kinase and inactivation of the X chromosome. Science 1972; 175: 1002-1003.
69. Davidson RG, Nitowsky HM, Childs B. Demonstration of Two Populations of Cells in the Human Female Heterozygous for Glucose-6-Phosphate Dehydrogenase Variants. Proc Natl Acad Sci U S A 1963; 50: 481-485.
70. Migeon BR, Der Kaloustian VM, Nyhan WL, Yough WJ, Childs B. X-linked hypoxanthine-guanine phosphoribosyl transferase deficiency: heterozygote has two clonal populations. Science 1968; 160: 425-427.
71. Gartler SM, Chen SH, Fialkow PJ, Giblett ER, Singh S. X chromosome inactivation in cells from an individual heterozygous for two X-linked genes. Nat New Biol 1972; 236: 149-150.
72. Patrat C, Okamoto I, Diabangouaya P, Vialon V, Le Baccon P, Chow J, Heard E. Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. Proc Natl Acad Sci U S A 2009; 106: 5198-5203.
73. Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E. Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 2004; 303: 644-649.
74. Okamoto I, Heard E. Lessons from comparative analysis of X-chromosome inactivation in mammals. Chromosome Res 2009; 17: 659-669.
75. Miller AP, Willard HF. Chromosomal basis of X chromosome inactivation: identification of a multigene domain in Xp11.21-p11.22 that escapes X inactivation. Proc Natl Acad Sci U S A 1998; 95: 8709-8714.
76. Huynh KD, Lee JT. Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. Nature 2003; 426: 857-862.
77. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984; 37: 179-183.
78. Davies W, Isles AR, Wilkinson LS. Imprinted gene expression in the brain. Neurosci Biobehav Rev 2005; 29: 421-430.
79. Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat Rev Genet 2001; 2: 21-32.
80. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N. Reactivation of the paternal X chromosome in early mouse embryos. Science 2004; 303: 666-669.
81. Latham KE, Rambhatla L. Expression of X-linked genes in androgenetic, gynogenetic, and normal mouse preimplantation embryos. Dev Genet 1995; 17: 212-222.
82. Barlow DP. Methylation and imprinting: from host defense to gene regulation? Science 1993; 260: 309-310.
83. Emerson JJ, Kaessmann H, Betran E, Long M. Extensive gene traffic on the mammalian X chromosome. Science 2004; 303: 537-540.
84. Skuse DH, James RS, Bishop DV, Coppin B, Dalton P, Aamodt-Leeper G, Bacarese-Hamilton M, Creswell C, McGurk R, Jacobs PA. Evidence from Turner's syndrome of an imprinted X-linked locus affecting cognitive function. Nature 1997; 387: 705-708.
85. Bishop DV, Canning E, Elgar K, Morris E, Jacobs PA, Skuse DH. Distinctive patterns of memory function in subgroups of females with Turner syndrome: evidence for imprinted loci on the X-chromosome affecting neurodevelopment. Neuropsychologia 2000; 38: 712-721.
86. Kesler SR, Blasey CM, Brown WE, Yankowitz J, Zeng SM, Bender BG, Reiss AL. Effects of X-monosomy and X-linked imprinting on superior temporal gyrus morphology in Turner syndrome. Biol Psychiatry 2003; 54: 636-646.
87. Stemkens D, Roza T, Verrij L, Swaab H, van Werkhoven MK, Alizadeh BZ, Sinke RJ, Giltay JC. Is there an influence of X-chromosomal imprinting on the phenotype in Klinefelter syndrome? A clinical and molecular genetic study of 61 cases. Clin Genet 2006; 70: 43-48.
88. Nguyen DK, Disteche CM. Dosage compensation of the active X chromosome in mammals. Nat Genet 2006; 38: 47-53.

89. Zechner U, Wilda M, Kehrer-Sawatzki H, Vogel W, Fundele R, Hameister H. A high density of X-linked genes for general cognitive ability: a run-away process shaping human evolution? Trends Genet 2001; 17: 697-701.
90. Tarpey PS, Smith R, Pleasance E, Whibley A, Edkins S, Hardy C, O'Meara S, Latimer C, Dicks E, Menzies A, Stephens P, Blow M, Greenman C, Xue Y, Tyler-Smith C, Thompson D, Gray K, Andrews J, Barthorpe S, Buck G, Cole J, Dunmore R, Jones D, Maddison M, Mironenko T, Turner R, Turrell K, Varian J, West S, Widaa S, Wray P, Teague J, Butler A, Jenkinson A, Jia M, Richardson D, Shepherd R, Wooster R, Tejada MI, Martinez F, Carvill G, Goliath R, de Brouwer AP, van Bokhoven H, Van Esch H, Chelly J, Raynaud M, Ropers HH, Abidi FE, Srivastava AK, Cox J, Luo Y, Mallya U, Moon J, Parnau J, Mohammed S, Tolmie JL, Shoubridge C, Corbett M, Gardner A, Haan E, Rujirabanjerd S, Shaw M, Vandeleur L, Fullston T, Easton DF, Boyle J, Partington M, Hackett A, Field M, Skinner C, Stevenson RE, Bobrow M, Turner G, Schwartz CE, Gecz J, Raymond FL, Futreal PA, Stratton MR. A systematic, large-scale resequencing screen of X-chromosome coding exons in mental retardation. Nat Genet 2009; 41: 535-543.
91. Skuse DH. Imprinting, the X-chromosome, and the male brain: explaining sex differences in the liability to autism. Pediatr Res 2000; 47: 9-16.
92. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, Chinwalla A, Delehaunty A, Delehaunty K, Du H, Fewell G, Fulton L, Fulton R, Graves T, Hou SF, Latrielle P, Leonard S, Mardis E, Maupin R, McPherson J, Miner T, Nash W, Nguyen C, Ozersky P, Pepin K, Rock S, Rohlfing T, Scott K, Schultz B, Strong C, Tin-Wollam A, Yang SP, Waterston RH, Wilson RK, Rozen S, Page DC. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 2003; 423: 825-837.
93. Tronchere H, Wang J, Fu XD. A protein related to splicing factor U2AF35 that interacts with U2AF65 and SR proteins in splicing of pre-mRNA. Nature 1997; 388: 397-400.
94. Zhang Z, Joh K, Yatsuki H, Wang Y, Arai Y, Soejima H, Higashimoto K, Iwasaka T, Mukai T. Comparative analyses of genomic imprinting and CpG island-methylation in mouse Murr1 and human MURR1 loci revealed a putative imprinting control region in mice. Gene 2006; 366: 77-86.
95. Su WL, Modrek B, GuhaThakurta D, Edwards S, Shah JK, Kulkarni AV, Russell A, Schadt EE, Johnson JM, Castle JC. Exon and junction microarrays detect widespread mouse strain- and sex-bias expression differences. BMC Genomics 2008; 9: 273.
96. Lu H, Huang X, Zhang L, Guo Y, Cheng H, Zhou R. Multiple alternative splicing of mouse Dmrt1 during gonadal differentiation. Biochem Biophys Res Commun 2007; 352: 630-634.
97. Schutt C, Nothiger R. Structure, function and evolution of sex-determining systems in Dipteran insects. Development 2000; 127: 667-677.
98. Ferguson-Smith M. The evolution of sex chromosomes and sex determination in vertebrates and the key role of DMRT1. Sex Dev 2007; 1: 2-11.
99. Just W, Rau W, Vogel W, Akhverdian M, Fredga K, Graves JA, Lyapunova E. Absence of Sry in species of the vole Ellobius. Nat Genet 1995; 11: 117-118.

CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

1) Timing of gamete interaction and maturity of the oocyte at the time of the interaction can affect the kinetics of early cleavage divisions but has no effect on the sex ratio of the embryos produced.

2) *In vitro* matured oocytes are not able to preferentially select sperm of one sex over another.

3) The use of sex-sorted semen produces a reduction in fertility following IVF, which is caused by the sperm damage caused by the sorting procedure. This reduction in fertility is associated with a delay in the timing of the first cleavage, which differs between bulls. However, no effect on the timing of blastocysts appearance and on gene expression at the blastocyst stage is observed.

4) There are no significant differences in the kinetics of the first cleavage and blastocysts appearance and in the survival in culture conditions between male and female embryos produced with sorted or unsorted semen.

5) Increased sex ratios are obtained following IVF with sorted or unsorted semen from different bulls, which suggests a slightly higher fertilization ability for Y-bearing spermatozoa.

6) There is a higher transcriptional level for the epigenetic-related enzymes *DNMT3A*, *DNMT3B*, *HMT1* and *ILF3*, and a higher methylation level for the sequence *VNTR* in male embryos compared with females, suggesting a higher transcriptional level in female embryos.

7) Male embryos content higher mtDNA copy number and shorter telomere length than females.

8) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Gene ontology analysis of the sexually dimorphic transcripts suggests a higher transcriptional level for female blastocysts and a

more active protein metabolism and an increased mitochondrial activity for males.

9) Most X-linked expressed transcripts (88.5 %) are upregulated in females, but most of them (70 %) exhibited fold changes lower than 1.6 suggesting that X-chromosome inactivation is partially achieved at the blastocyst stage. Five X-linked genes (*BEX*, *CAPN6*, *BEX2*, *SRPX2* and *UBE2A*) are preferentially expressed by the paternal X-chromosome. Imprinting mechanisms may increase the transcriptional skew caused by double X-chromosome dosage.

10) A novel gene (*YZRSR2*), homologue to an X-linked gene involved in network interactions during spliceosome assembly, was discovered in the bovine Y-chromosome.

CONCLUSIONES

1) El tiempo de interacción entre gametos y el grado de maduración del ovocito en el momento de la interacción puede afectar a la cinética de las divisiones tempranas, pero no altera la proporción de sexos de los embriones generados.

2) Los ovocitos madurados *in vitro* no son capaces de seleccionar al espermatozoide portador de un sexo determinado.

3) El uso de semen sexado da lugar a una reducción de la fertilidad tras la FIV, debida al daño espermático producido por el procedimiento de separación espermática. Esta reducción de la fertilidad está asociada a un retraso en el tiempo de la primera división, que varía en función del toro. Sin embargo, la cinética de desarrollo a blastocisto y la expresión génica en el estadio de blastocisto no se ven afectados.

4) No hay diferencias significativas entre embriones macho y hembra producidos con semen sexado o sin sexar en la cinética de la primera división, en el desarrollo a blastocisto y en la supervivencia en condiciones de cultivo.

5) La proporción de sexos obtenida tras la FIV con semen sexado o sin sexar de diferentes toros es superior a 1:1, lo que sugiere una capacidad fecundante superior en los espermatozoides Y.

6) Los embriones macho presentan un mayor nivel de transcripción de las enzimas relacionadas con la epigenética *DNMT3A*, *DNMT3B*, *HMT1* e *ILF3*, y un mayor nivel de metilación en la secuencia *VNTR* en comparación con las hembras, lo que sugiere un mayor nivel transcripcional en embriones hembra.

7) Los embriones macho contienen más copias de ADN mitocondrial y telómeros más cortos que los embriones hembra.

8) El sexo determina el nivel de expresión de una tercera parte de los genes expresados en los blastocistos bovinos. El análisis de ontología génica de los

transcritos que muestran dimorfismo sexual sugiere un nivel de transcripción más alto en los blastocistos hembras y un mayor metabolismo proteico y actividad mitocondrial en los machos.

9) La mayoría de los transcritos expresados ligados al cromosoma X (88,5 %) están sobreexpresados en hembras, pero la mayoría (70 %) muestran cambios de expresión inferiores a 1,6 sugiriendo una inactivación parcial del cromosoma X en el estadio de blastocisto. Cinco genes ligados al cromosoma X (*BEX*, *CAPN6*, *BEX2*, *SRPX2* and *UBE2A*) se expresan preferencialmente mediante el cromosoma X paterno. Los mecanismos de impronta genómica parental pueden incrementar el sesgo transcripcional causado por la doble dosis de cromosoma X.

10) Se ha descubierto un nuevo gen (*YZRSR2*) presente en el cromosoma Y bovino, homólogo a un gen ligado al cromosoma X e implicado en la red de interacciones del ensamblado del espliceosoma.

ABBREVIATIONS/ABREVIATURAS

ABBREVIATIONS

ACTH: Adrenocorticotropic hormone.
AI: Artificial insemination.
AMV: Avian myeloblastosis virus.
ANOVA: Analysis of variance.
ATLR: Average telomere lenght ratio.
B.C.: Before Christ.
bp: Base pairs.
BSA: Bovine serum albumin.
BWS: Beckwith-Wiedemann syndrome.
CA: California.
cDNA: Complementary deoxyribonucleic acid.
Chr: Chromosome.
COC: Cumulus oocyte complex.
CT: Cycle threshold.
DMAP: Dimethylaminopurine.
DMR: Differentially methylated region.
DNA: Deoxyribonucleic acid.
Dnmts: DNA methyltransferases.
dNTP: Deoxyribonucleotide tri-phosphate.
E: Embryonic day.
EDTA: Ethylenediaminetetraacetic acid.
e.g.: *Exempli gratia* (For example).
EGCs: Embryonic germ cells.
EGA: Embryonic genome activation.
ES: Embryonic stem (cells).
F: Female.
FCS: Fetal calf serum.
FDR: False discovery rate.
FF: Follicular fluid.
GlcNAc: N-Acetylglucosamine.
h.: Hours.
hpi: Hours post-insemination.
Hya: Histocompatibility Y antigen.
ICSI: Intracytoplasmic sperm injection.
ICM: Inner cell mass.
ID: Identification (number).
i.e: *Id est* (that is).
IU: International unit.
IVF: *In vitro* fertilization.
IVM: *In vitro* maturation.
Kb: Kilobases.
LH: Luteinizing hormone.
LOS: Large offspring syndrome.
M: Male.
Mb: Megabases.
MES: 2-(N-morpholino)ethanesulfonic acid.
Min: Minutes.
MMLV: Moloney murine leukemia virus.

mRNA: Messenger ribonucleic acid.
mtDNA: Mitochondrial deoxyribonucleic acid
NADPH: Nicotinamide adenine dinucleotide phosphate.
OR: Oregon.
OSR: Operational sex ratio.
PBS: Phosphate buffered saline.
PCR: Polymerase chain reaction.
PGCs: Primordial germ cells.
PPP: Pentose phosphate pathway.
PUFA: Polyunsaturated fatty acids.
qPCR, qRT-PCR or RT-PCR: Quantitative (real time) polymerase chain reaction.
RMA: Robust multi-array.
RNA: Ribonucleic acid.
rRNA: Ribosomal ribonucleic acid.
RT: Retrotranscription.
s or sec: Seconds.
s.e.m or SE: Standard error of the mean.
SI: Supporting information.
SINE: Short interspersed nuclear element.
SOF: Synthetic oviduct fluid.
SSPs: Sex-specific proteins.
TCM-199: Tissue culture medium 199.
TEST (buffer): Buffer made up of TRIS, EDTA and NaCl.
TRIS: Tris(hydroxymethyl)aminomethane.
UK: United Kingdom.
USA: United States of America.
US$: United States of America dollars.
UV: Ultraviolet.
v. or vs.: *Versus*.
VNTR: Variable number of tandem repeats.
WI: Wisconsin.
XCI: X-chromosome inactivation.

Physical units (volume, weight, voltage, gravity…) are abbreviated following the International System of Units. Chemical substances are named following the IUPAC (International Union of Pure and Applied Chemistry) nomenclature.

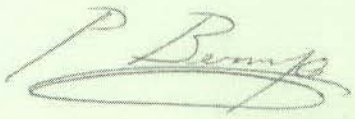
Genes and proteins are named by their symbols following the nomenclature of the National Center for Biotechnology Information´s (http://www.ncbi.nlm.nih.gov/sites/entrez).

ABREVIATURAS

A.C.: Antes de Cristo.
ACTH: Hormona adrenocorticotropa.
ADN: Ácido desoxirribonucleico.
ARNm: Ácido ribonucleico mensajero.
CGE: Células geminales embrionarias.
CGP: Células germinales primordiales.
hpi: Horas postinseminación.
IA: Inseminación artificial.
ICSI: Inyección intracitoplasmática de un espermatozoide.
ICX: Inactivación del cromosoma X.
FIV: Fecundación *in vitro*.
LH: Hormona luteinizante.
NADPH: Nicotinamida adenina dinucleótido fosfato.
qPCR: Reacción en cadena de la polimerasa cuantitativa (a tiempo real).
RMD: Región metilada de forma diferencial.

Los genes y las proteinas han sido nombrados por sus símbolos siguiendo la nomenclatura del NCBI (Centro nacional de informaciones biotecnológicas) (http://www.ncbi.nlm.nih.gov/sites/entrez).

CURRICULUM VITAE



Education

- DVM, Complutense University, Madrid, Spain, 2005.
- Veterinary graduate ("Título de grado"), Complutense University, Madrid, Spain, 2005.
- Veterinary Sciences Research Degree ("DEA"), Complutense University, Madrid, Spain, 2007.
- Languages knowledge: English and basic French.

Working history (science)

- Pregraduate scholarship "Beca de colaboración UCM" (Complutense University). Animal Physiology Department, Veterinary Faculty, UCM (Spain). October 2004-June 2005.
- Pregraduate scholarship "EU-FIPSE" (European Union). Kleberg Center, Texas A&M University (USA). July-September 2005.
- Postgraduate scholarship "FPU" (Spanish Ministry of Education). Animal Reproduction Department, INIA (Spain). April 2006-April 2008.
- FPU contract (Spanish Ministry of Education. Animal Reproduction Department, INIA (Spain). April 2008-at present.
- Specialty (UNESCO codes): 240107, 240901. Research lines: Physiology of Reproduction, Molecular Embryology and Assisted Reproduction in Domestic Animals.

Articles in indexed journals

- "Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts". **Bermejo-Álvarez P**, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. Proceedings of the National Academy of Sciences. In press. 2010.
- "Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability" Sturmey RG, **Bermejo-Álvarez P** , Gutiérrez-Adán A, Rizos D, Leese HJ, Lonergan P. Molecular Reproduction and Development; 77(3):285-296. 2010.
- "Intrafollicular testosterone concentration and sex ratio in individually cultured bovine embryos" García-Herreros M, **Bermejo-Álvarez P**, Rizos D, Gutiérrez-Adán A, Fahey AG, Lonergan P. Reproduction, Fertility and Development. In press. 2010.
- "Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolysis" **Bermejo-Álvarez P** , Lonergan P, Rizos D, Gutiérrez-Adán A. Reproductive Biomedicine Online. In press. 2010.
- "Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa" **Bermejo-Álvarez P**, Lonergan P, Rath D, Gutiérrez-Adán A, Rizos D. Reproduction, Fertility and Development; 22(2):426-436. 2010.
- "Gene expression in early expanded parthenogenetic and in vitro fertilized bovine blastocysts" Gómez E, Caamaño JN, **Bermejo-Álvarez P** , Díez C, Muñoz M, Martín D, Carrocera S, Gutiérrez-Adán A. Journal of Reproduction and Development; 55(6):607-614. 2010.

- "Changes in testosterone or temperature during the in vitro oocyte culture do not alter the sex ratio of bovine embryos" Díez C, **Bermejo-Álvarez P**, Trigal B, Caamaño JN, Muñoz M, Molina I, Gutiérrez-Adán A, Carrocera S, Martín D, Gómez E. Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology; 311(6):448-452. 2009.
- "Biological differences between in vitro produced bovine embryos and parthenotes" Gómez E, Gutiérrez-Adán A, Díez C, **Bermejo-Álvarez P** , Muñoz M, Rodríguez A, Otero J, Álvarez-Viejo M, Carrocera S, Martín D, Caamaño J. Reproduction; 137(2):285-295. 2009.
- "Consequences of in vitro culture conditions on embryo development and quality" Rizos D, Clemente M, **Bermejo-Alvarez P**, de La Fuente J, Lonergan P, Gutiérrez-Adán A. Reproduction of Domestic Animals; 43(S4):44-50. 2008.
- "Effect of duration of oocyte maturation on the kinetics of cleavage, embryo yield and sex ratio in cattle" Rizos D, **Bermejo-Álvarez P**, Gutiérrez-Adán A, Lonergan P. Reproduction, Fertility and Development; 20(6):734-740. 2008.
- "Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially?" **Bermejo-Álvarez P** , Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. Biology of Reproduction; 79(4):594-597. 2008
- "Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring" Fernández-González R, Moreira P, Pérez-Crespo M, Sánchez-Martín M, Ramírez MA, Pericuesta E, Bilbao A, **Bermejo-Alvarez P** , Hourcade JD, Rodríguez de Fonseca F, Gutiérrez-Adán A. Biology of Reproduction; 78(4):761-772. 2008.
- "Epigenetic differences between male and female blastocyst produced in vitro" **Bermejo-Álvarez P**, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. Physiological Genomics; 32(2):264-272. 2008.

Other publications

- "Telomere lengthening from oocyte to embryonic stem cell" Chapter of the book "Telomeres: Function, Shortening and Lenghtening" Ed. Nova Publishers. 2009.
- "Telomere lengthening during preimplantation development" Pericuesta E, Ramírez MA, Fernández-González R, Pérez-Crespo M, Hourcade JD, **Bermejo P**, Rizos D, Gutiérrez-Adán A. Association Europeenne de transfert embryonnaire A.E.T.E. Newsletter; 29:10-14. 2007.

Research stays

- Kleberg Center, University of Texas A&M (USA). Dr. Fuller Bazer laboratory. 2 months, July-September 2005. Research focussed on pregnancy signalling in ovine.
- Lyons Research Farm, University College Dublin (Ireland). Dr. Pat Lonergan laboratory. 4 months, August-December 2007, plus 1 month in March and December 2008. Research focussed on bovine in vitro and in vivo embryo production.

- Center for Regenerative Biology, University of Connecticut (USA). Dr. Cindy Tian laboratory. 4 months, June-October 2008. Research focussed on SCNT in mouse and bovine.
- School of Biosciences, University of Nottingham (United Kingdom). Dr. Keith Campbell laboratory. 4 months, July-October 2009. Research focussed on SCNT in ovine.

Oral communications in international scientific meetings

- "Identification of five genes expressed preferentially from the paternal X chromosome" **Bermejo-Alvarez P** , Rizos D, Lonergan P, Gutiérrez-Adán. 36th Annual Conference of the International Embryo Transfer Society (IETS). Córdoba, Argentina, USA. January 2010.
- "Micro array analysis reveals that one third of the genes actively expressed are differentially expressed between male and female bovine blastocysts". **Bermejo-Álvarez P** , Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. 42nd Annual Meeting of the Society for the Study of Reproduction (SSR). Pittsburgh, USA. July 2009.
- "Effect of oxygen tension during in vitro maturation and fertilization on bovine embryo production and cumulus-oocyte-complex mRNA abundance". **Bermejo-Álvarez**, Rizos D, Lonergan P, Gutiérrez-Adán A. 35th Annual Conference of the International Embryo Transfer Society (IETS). San Diego, USA. January 2009.
- "Differences in methylation status between male and female bovine blastocysts produced in vitro" **Bermejo-Álvarez P** , Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. 23rd Scientific Meeting of the European Embryo Transfer Society (AETE). Alghero, Italy. September 2007.

Poster presentations in international scientific meetings as first author

- "mtDNA copy number during bovine preimplantation development". **Bermejo-Álvarez P**, Rizos D, Lonergan P, Gutiérrez-Adán A. Cost Action – Gemini "Maternal Interactions with Gametes and Embryos". Alghero, Italy. October 2009.
- "Bovine sex ratio is not altered following three different modifications in IVF protocol". **Bermejo-Álvarez P**, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. 25th Scientific Meeting of the European Embryo Transfer Society (AETE). Poznan, Poland. September 2009.
- "Sex ratio in bovine preimplantation embryos produced either in vitro or in vivo". **Bermejo-Álvarez P**, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. Cost Action – Gemini "Maternal Interactions with Gametes and Embryos". Lansk, Poland. May 2009.
- "Effects of oxygen tension during in vitro maturation and fertilization in bovine embryo production" **Bermejo-Álvarez P** , Rizos D, Lonergan P, Gutiérrez-Adán A 1st International Congress of the Spanish Society for Animal Reproduction (AERA) and British Andrology Society (BAS). Gijón, Spain. October 2008.
- "Developmental kinetics of male and female bovine embryos produced in vitro with sex-sorted sperm" **Bermejo-Álvarez P** , Lonergan D, Rath A,

Gutiérrez-Adán A, Rizos D 24th Scientific Meeting of the European Embryo Transfer Society (AETE). Pau, France. September 2008.

- "Can bovine in vitro matured oocytes process differentially X- or Y-bearing spermatozoa?" **Bermejo-Álvarez P**, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. 41st Annual Meeting of the Society for the Study of Reproduction (SSR). Lailua-Lona, USA. May 2008.
- "Does duration of bovine oocyte maturation affect the speed of embryo development in in vitro and sex ratio at the two-cell or blastocyst stage?" **Bermejo-Álvarez P** , Gutiérrez-Adán A, Lonergan P, Rizos D. 34th Annual Conference of the International Embryo Transfer Society (IETS). Denver, USA. January 2008

Other contributions in international meetings

- Poster: "Sex-dependent metabolic differences of bovine preimplantation embryos" Sturmey RG, **Bermejo-Álvarez** P, Gutiérrez-Adán A, Rizos D, Leese HJ, Lonergan P. 36th Annual Conference of the International Embryo Transfer Society (IETS). Córdoba, Argentina, USA. January 2010.
- Poster: "Relative mRNA abundance in ovine blastocysts produced in vivo or in vitro in different culture media". Sanna D, **Bermejo-Álvarez P** , Mara L, Rizos D, Gutiérrez-Adán A, Dattena M. 25th Scientific Meeting of the European Embryo Transfer Society (AETE). Poznan, Poland. September 2009.
- Oral communication: "Relationship between follicle size, intrafollicular testosterone concentration and sex ratio in individually cultured bovine embryos". García-Herreros M, **Bermejo-Álvarez P**, Rizos D, Gutiérrez-Adán A, Fahey AH, Lonergan P. 25th Scientific Meeting of the European Embryo Transfer Society (AETE). Poznan, Poland. September 2009.
- Poster: "Testosterone in the oocyte culture does not alter sex-ratio of in vitro produced bovine embryos". Díez C, **Bermejo-Álvarez P**, Gutiérrez-Adán A, Caamaño JN, Muñoz M, Carrocera S, Martín D, Gómez E. 35th Annual Conference of the International Embryo Transfer Society (IETS). San Diego, USA. January 2009.
- Poster: "Sex ratio in bovine embryos is not regulated by temperature incubation of oocytes" Díez C, **Bermejo-Álvarez P** , Gutiérrez-Adán A, Caamaño JN, Muñoz M, Carrocera S, Martin D, Gómez E. 1st International Congress of the Spanish Society for Animal Reproduction (AERA) and British Andrology Society (BAS). Gijón, Spain. October 2008.
- Poster "Timing of blastocysts expansion in bovine IVF embryos and parthenotes affects expression of genes involved in pluripotency, methylation, apoptosis and pregnancy recognition" Caamaño JN, Muñoz M, Gutiérrez-Adán A, **Bermejo-Álvarez P**, Díez C, Otero J, Álvarez-Viejo M, Carrocera S, Martin D, Gómez E. 1st International Congress of the Spanish Society for Animal Reproduction (AERA) and British Andrology Society (BAS). Gijón, Spain. October 2008.
- Poster: "Expression of genes involved in compactation, blastulation and metabolism in bovine IVF embryos and parthenotes depends on timing of blastocysts expansion" Gómez E, **Bermejo-Álvarez P**, Caamaño, JN, Muñoz M, Díez C, Carrocera S, Martin D, Rodríguez A, Gutiérrez-Adán A 24th

Scientific Meeting of the European Embryo Transfer Society (AETE). Pau, France. September 2008.

- Poster: "Effect of exogenous leptin on in vitro development of bovine embryos" Arias-Álvarez M, **Bermejo-Álvarez P** , Rizos D, Lorenzo PL, Gutiérrez-Adán A, Lonergan P 24th Scientific Meeting of the European Embryo Transfer Society (AETE). Pau, France. September 2008.
- Oral Communication: "Use of Axinfu mutation model to analyze preimplantation epigenetic alterations produced by in vitro culture in mice" Fernández-González R, Pérez-Crespo M, **Bermejo P** , De Dios J, Pintado B, Gutiérrez-Adán A. 2nd International Meeting of Mammalian Embryogenomics. Paris, France. October 2007.
- Oral communication: "Effects of BSA and hyaluronan in in vitro culture of ovine embryos and their quality in terms of cryotolerance, gene expression, lambing rate and birth weight" Sanna S, Rizos D, Mara L, **Bermejo-Álvarez P**, Gutiérrez-Adán A, Dattena M. 23rd Scientific Meeting of the European Embryo Transfer Society (AETE). Alghero, Italy. September 2007.
- Oral communication: "Inheritance of epigenetic alterations produced by in vitro culture in mice" Fernández-González R, Ramirez MA, Pérez-Crespo M, Pericuesta E, **Bermejo P** , Hourcade JD, Pintado B, Gutiérrez-Adán A. 23rd Scientific Meeting of the European Embryo Transfer Society (AETE). Alghero, Italy. September 2007.
- Oral communication: "Transgenerational epigenetic alterations in mice produced by in vitro culture" Fernández-González R, Ramírez MA, Pérez-Crespo M, Pericuesta E, **Bermejo P**, Hourcade JD, Pintado B, Gutiérrez-Adán A. 40th Annual Meeting of the Society for the Study of Reproduction (SSR). San Antonio, USA. July 2007.
- Poster: "Unexpected severe abnormalities in mouse ROSI offspring" Moreira PN, Fernández-González R, Pérez-Crespo M, **Bermejo P**, Hourcade JD, Rey R, Gutiérrez-Adán A. 33th Annual Conference of the International Embryo Transfer Society (IETS). Kyoto, Japón. January 2007.
- Poster: "Effects of maturation stage of bovine oocytes on sex ratio and speed of embryo development" Rizos D, **Bermejo P**, Gutiérrez-Adán A, Lonergan, P. 22nd Scientific Meeting of the European Embryo Transfer Society (AETE). Zug, Suiza. September 2006.
- Poster: "Progesterone levels during 20 days of pregnanty of rabbit treated for endometriosis or with anti-CD44" Illera MJ, **Bermejo P** , Natarajan A, Willingham C, Hernández J, González A, Silván G, Illera JC. Copenhague, Denmark. January 2005.
- Poster: "The effect of anti-CD44 on embryo implantation in rabbits" Illera MJ, **Bermejo P** , Hernández J, González A, Illera JC. Portland, USA. January 2004.

Assistance to international meetings

- 36th Annual Conference of the International Embryo Transfer Society (IETS). Travel grant. Córdoba, Argentina, USA. January 2010.
- COST-GEMINI "Maternal interaction with gametes and embryos". Local Comitee Grant. Alguero, Italy. October 2009.

- 42nd Annual Meeting of the Society for the Study of Reproduction (SSR). Pittsburgh, USA. July 2009.
- COST-GEMINI "Maternal interaction with gametes and embryos". Student Special Grant. Lansk, Poland. May 2009.
- 35th Annual Conference of the International Embryo Transfer Society (IETS). Travel grant. San Diego, USA. January 2009.
- 1st International Congress of the Spanish Society for Animal Reproduction (AERA) and British Andrology Society (BAS). Gijón, Spain. October 2008.
- 23nd Scientific Meeting of the European Embryo Transfer Society (AETE). Alghero, Italy. September 2007.

Contributions and assistance to National Meetings

- "Efectos del antiCD44 sobre la implantación y los niveles hormonales de la coneja". II Congreso Europeo de Investigación en Pregrado de Ciencias de la Salud. Madrid, 2005.
- "Influencia de las combinaciones anestésicas ketamina-xilacina y ketamina-xilacina-buprenorfina sobre la función adrenal en el conejo"; "Efectos de la ampliación de la UE sobre la política agraria española"; "Niveles de esteroides sexuales durante la primera mitad de la gestación en la coneja endometriosa"; "Efectos del antiCD44 sobre la implantación embrionaria y la curva de progesterona en la coneja". IV Congreso de ciencias veterinarias y biomédicas. Madrid, 2005.
- "Niveles hormonales de la coneja endometriosa durante el primer tercio de la gestación", "Efectos del antiCD44 sobre la implantación y los niveles hormonales en la coneja". III Congreso de Ciencias Veterinarias y Biomédicas. Madrid, 2004.
- "Efectos de la anestesia intravenosa e inhalatoria sobre la función adrenal en conejos NZW". II Congreso de Ciencias Veterinarias y Afines. Madrid, 2003.
- "Influencia del Estrés en la Reproducción Animal en Hembras" I Congreso de Ciencias Veterinarias y Afines. Madrid, 2002.

Awards

- Best oral communication in "IV Congreso de Ciencias Veterinarias y Biomédicas" Madrid, Spain. April 2005.
- First prize in Student Competition in 23nd Scientific Meeting of the European Embryo Transfer Society (AETE). Alghero, Italy. September 2007.
- Finalist in Student Competition in 35th Annual Conference of the International Embryo Transfer Society (IETS). San Diego, USA. January 2009.
- Finalist in Student Competition in 36th Annual Conference of the International Embryo Transfer Society (IETS). Córdoba, Argentina. January 2009.

Teaching

- XXXII International Course of Animal Reproduction, INIA, Madrid, Spain. November 2009.
- XXXI International Course of Animal Reproduction, INIA, Madrid, Spain. November 2008.

PABLO BERMEJO ÁLVAREZ TESIS DOCTORAL

2010