Universitat de Girona

IN VITRO PRODUCTION OF PORCINE EMBRYOS. NEW INSIGHT INTO OPTIMIZATION OF CULTURE MEDIA WITH RELEVANCE TO SEX RATIO PURPOSES

Eva Torner Bosch

Dipòsit legal: Gi. 1453-2014
http://hdl.handle.net/10803/276958

Universitat de Girona

Doctoral Thesis

In Vitro Production of Porcine Embryos. New Insights into Optimization of Culture Media with Relevance to Sex Ratio Purposes

Eva Torner Bosch

2014

Universitat de Girona

Doctoral Thesis

In Vitro Production of Porcine Embryos. New Insights into Optimization of Culture Media with Relevance to Sex Ratio Purposes

Eva Torner Bosch
2014

Doctoral program in Technology

Supervised by:

Dr. SERGI BONET MARULL
Dra. MARIA DOLORS BRIZ GONZÁLEZ
Dra. EVA BUSSALLEU MUNTADA

Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy. University of Girona.

Universitat de Girona

El Dr. Sergi Bonet Marull, Catedràtic del Departament de Biologia de la Facultat de Ciències de la Universitat de Girona.

La Dra. Maria Dolors Briz González, Professora Titular del Departament de Biologia de la Facultat de Ciències de la Universitat de Girona.

La Dra. Eva Bussalleu Muntada, Professora Lectora del Departament de Biologia de la Facultat de Ciències de la Universitat de Girona.

DECLAREM:

Que el treball titulat "*In Vitro* Production of Porcine Embryos. New Insights into Optimization of Culture Media with Relevance to Sex Ratio Purposes", que presenta Eva Torner Bosch per a l'obtenció del títol de doctora per la Universitat de Girona, ha estat realitzat sota la nostra direcció.

I, perquè així consti i tingui els efectes oportuns, signem aquest document.

Dr. Sergi Bonet Marull Dra. Maria Dolors Briz González Dra. Eva Bussalleu Muntada

La doctoranda: Eva Torner Bosch

Girona, març 2014

The work of a thesis is a process, not an event; it is about the journey, not only the destination. And this journey is as much a journey of self-learning and personal transformation as it is an academic exploration. It is about developing one's identity as a researcher and writer, gaining confidence and trust in oneself, finding voice.

- Ardra L. Cole

The Thesis Journey: Travelling with Charley
Brock Education (2003), 13(1):1-13

Always remember, a cat looks down on man, a dog looks up to man, but a pig will look man right in the eye and see his equal.

- Winston Churchill

To my grandparents.

To my parents, for always being there.

Acknowledgments

It may sound cliché to think of the doctoral thesis as a journey, but when one finishes and looks back one agrees it is a good analogy. Now, the journey of my doctoral thesis, long and arduous, sometimes solitary, and intensely personal, has ended. But it has not been a journey into emptiness. I eagerly connected with fellow travellers with whom I shared unforgettable experiences, common interests and hard work. Those precious connections and the support of my beloved ones have encouraged me to hold on. Thus, besides my efforts, the success of this journey has depended largely on the inspiration, guidance and patience of several colleagues who have accompanied me on different part of the journey, and to whom I owe my deepest gratitude.

I would specially like to thank Sergi Bonet. You offered me the boarding pass to start the journey and provided me with an excellent working atmosphere and infrastructure to get the destination. I am also thankful to you, Mailo Briz and Eva Bussalleu, for your viewpoint and comments on my work, your trust and confidence, and for giving me the enormous freedom to choose my own research topics and ideas. I am also grateful to Beth Pinart and Sílvia Sancho. Your helpful advice, both professional and personal, was always granted with a big smile. Also, I am indebted to Marc Yeste for the unconditional support. Continually and persuasively you conveyed a spirit of adventure in my research so that I felt motivated and encouraged after our meetings. Thank you for helping me grow into a research scientist.

Sincere appreciation is extended to the members and former members of Technosperm: Eva, Marc, Isabel, Anna, Mònica, Marta, Estela, Miriam, Lílian, Eva, Ingrid, Roser, Albert and Noelia. Thank you for providing a stimulating and fun-filled environment. Thank you for comforting me in the difficult moments along the way and for always conveying a positive thought. Thank you for helping me to choose the best path and for always being there. You have become more than fellow travellers.

Most of the goals achieved in this journey would not have been accomplished without collaborating with other laboratories. I owe a great deal of appreciation and gratitude to Teresa Mogas and Roser Morató (Department of Animal Medicine and Surgery, UAB); Alfonso Gutiérrez and Ricardo Laguna (Department of Animal Reproduction, INIA); Pilar Coy, Carmen Matás and Lenka Tumova (Department of Physiology, University of Murcia); and Josep Santaló, Joaquima Navarro and Laia Ramos (Department of Cell

Biology, Physiology and Immunology, UAB). Thank you for opening the doors of your laboratories and for providing me guidance and hands-on-training on different aspects of my research.

I would also like to thank Sara, Ferran, Anna, Yolanda and the staff in the Faculty of Science, in the Institute of Food and Agricultural Technology (INTEA), and in the Scientific and Technological Park of the University of Girona. My gratitude goes also to Frigorífics del Ter and Frigorífics Cornellà, particularly to Joan, Laura and Marga, for selflessly providing the samples and always receiving us with a special greeting.

I am also indebted to Marta, Eloi, Natàlia, Anna, Eli, Nuri, Mireia, Gemma, Irene C., Clara and Irene V. It has been a pleasure to encounter some of you along the way. Thank you for being by my side throughout this journey. The journey is easier when we travel together.

My deepest and warmest appreciations go out to my family. This experience would not have been possible without your endless love, support and unwavering faith in me. My special love and thanks to my parents and grandparents for being by my side throughout my life. You have made me into who I am so this thesis is dedicated to you.

And last, but certainly not least, this thesis is also yours, Joan. Thank you for accompanying me on this adventure and the ones that will follow.

***

This work was supported by a FPU Grant (AP2008-01645) from the Spanish Ministry of Education and by a Pre-doctoral Grant (BR09/07) from the University of Girona.

Abbreviations & Symbols

<	Less than
>	Greater than
µl	Microlitre (10^{-3} ml)
µM	Micromolar
µm	Micrometre (10^{-3} mm)
A.D.	After death
AI	Artificial insemination
AMEL	Amelogenin
ATP	Adenosine triphosphate
BAX	Apoptosis regulator
B.C.	Before Christ
BECM	Beltsville embryo culture medium
BME	Basal medium Eagle's
bp	Base pair
BSA	Bovine serum albumin
BSA-V	Bovine serum albumin fraction V
Ca^{2+}	Calcium ion
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CCNB2	Cyclin B2
CD46	Membrane cofactor protein-46
CD55	Human decay-accelerating factor-55
CD59	Prolectin
CKS1B	Cyclin dependent kinase subunit 1
Cl^-	Chloride ion
CO_2	Carbon dioxide
CRP	Complement-regulatory protein
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3a
DNMT3B	DNA methyltransferase 3a
e.g.	*exempli gratia* (examples)
EAA	Essential amino acids
ECAD	Embryo cell-to-cell adhesion associated with embryo compactation
EDTA	Ethylenediaminetetraacetic acid
ET	Embryo transfer
EU	European Union
FAF-BSA	Fatty acid-free BSA
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridisation
G6PD	Glucose-6-phosphate dehydrogenase
GAG	Glycosaminoglycan
Gal	Galactose
gDNA	Genomic DNA
GLUT	Glucose transporter
GSH	Glutathione
h	Hour / hours
H2A	Histone 2A

H3A	Histone 3A
HA	Hyaluronic acid
hDAF	Human decay-accelerating factor
HPRT	Hypoxanthine phosphoribosyl transferase
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HMT1	hnRNP methyltransferase-like 2
IDH	Isocitrate dehydrogenase
IGF	Insulin growth factor
ILF3	Interleukin enhancer binding factor 3
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production / produced
K^+	Potassium ion
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
KSOM	Potassium simplex optimisation medium
MII	Metaphase II
MCP	Membrane cofactor protein
MEM	Minimum essential medium
Mg^{2+}	Magnesium ion
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar (mmol/liter)
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
mWM	Modified Whitten medium
Na^+	Sodium ion
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
NCSU	North Caroline State University
NEAA	Non-essential amino acids
nl	Nanolitre
ns-ET	Non- surgical embryo transfer
O_2	Oxygen
PA	Parthenogenetic-activated
pi	Post-insemination
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PO_4^{2-}	Phosphate ion
PP	Plant derived peptides
PPP	Pentose phosphate pathway
PTTG1	Pituitary tumour-transforming 1
PVA	Polyvinylalcohol
PVP	Polyvinylpyrolidone
PZM	Porcine zygote medium
RHAMM/IHABP	HA-mediated motility/intracellular HA-binding protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLBP	Stem-loop binding protein

SCNT	Somatic cell nuclear transfer
SO_4^{2-}	Sulfate ion
SOF	Synthetic oviductal fluid
SOX	Oxydative stress
Sry/SRY	Sex-determining region Y (mouse/human and other species)
TCA	Tricarboxylic acid
VNTR	Variable number of tandem repeats
vs.	Versus
XIAP	X-linked inhibitor of apoptosis protein
ZF	Zinc finger

List of Tables & Figures

Contents

List of Papers

This thesis is presented as a compendium of three papers:

Paper I

Torner E, Bussalleu E, Briz MD, Gutiérrez-Adán A, Bonet S. 2013. *Sex determination of porcine embryos using a new developed duplex polymerase chain reaction procedure based on the amplification of repetitive sequences.* Reproduction Fertility and Development 25(2):417-425.

Reproduction Fertility and Development has an impact factor of **2.583** and it is situated in the first quartile (**Q1**) in the Zoology category (© 2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

Paper II

Torner E, Bussalleu E, Briz MD, Yeste M, Bonet S. 2013. *Embryo development and sex ratio of in vitro-produced porcine embryos are affected by the energy substrate and hyaluronic acid added to the culture medium.* Reproduction Fertility and Development [In press] doi: 10.1071/RD13004

Reproduction Fertility and Development has an impact factor of **2.583** and it is situated in the first quartile (**Q1**) in the Zoology category (© 2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

Paper III

Torner E, Bussalleu E, Briz MD, Yeste M, Bonet S. 2013. *Energy substrate influences the effect of the timing of the first embryonic cleavage on the development of in vitro-produced porcine embryos in a sex-related manner.* Molecular Reproduction and Development 80(11):924-935.

Molecular Reproduction and Development has an impact factor of **2.812** and it is situated in the second quartile (**Q2**) in the Reproductive Biology category (© 2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

Abstract

This thesis deals with challenges that currently concern *in vitro* production (IVP), sex determination procedures and sex-related survival of porcine IVP embryos. Sex is considered one of the most important genetic traits for animal production. The ability to effectively select the desired sex of livestock offspring prior to conception has always been one of the main goals sought not only by breeders, but also by researchers. Sex preselection of IVP porcine embryos, together with cryopreservation and embryo transfer techniques, can be used to manipulate the sex ratio of offspring. This can improve the herd for desired purposes, reduce the generation interval, accelerate genetic improvement programmes and, thus, increase the biological and economic efficiency of productivity. Moreover, carrying out research on the selective loss of porcine male or female embryos under specific *in vitro* conditions will highlight novel physiological markers and molecular mechanisms responsible for sex ratio skews. In addition, it will provide the basis of how *in* vitro production can be enhanced in a sex-related manner. Nevertheless, these goals cannot be considered commercially applicable until the porcine IVP and sex determination procedures improve their efficiency.

With the aim of improving IVP techniques, two different approaches have been conducted to determine: (1) how the development of IVP porcine embryos is affected by replacing glucose with pyruvate and lactate in the culture medium for the first 48 h of culture; (2) how the addition of different concentrations of hyaluronic acid (HA) to the culture medium affect the development of IVP porcine embryos, and whether these putative effects of HA depend on the major energy substrate of the culture medium; and (3) how the development of IVP porcine embryos is affected by the timing of the first embryonic cleavage of embryos cultured with glucose- or pyruvate- and lactate-based media. Furthermore, both approaches have also been carried out to study sex-related embryo survival of embryos cultured under these conditions using a new developed duplex polymerase chain reaction (PCR) procedure.

First, and regardless of both the timing of the first embryonic cleavage and the presence of HA in the culture medium, results demonstrate that pyruvate and lactate at specific concentrations of 0.17 and 2.73 mM, respectively, are the preferred energy substrates at the early stages of porcine embryos. Embryos cultured with pyruvate-

lactate have significantly higher blastocyst rates than those cultured with 5.55 mM glucose. Moreover, culture with glucose decreases the kinetics of IVP embryo development. Second, it has been observed that the effects of HA on embryo development depend on its concentration and are not influenced by the energy substrate in the culture medium during the first 48 h post-insemination (pi). While 0.5 $mg \cdot ml^{-1}$ HA have no effect on embryo development, 1.0 $mg \cdot ml^{-1}$ HA significantly decreases blastocyst rates in both glucose- and pyruvate and lactate- based media. Finally, successful development to the blastocyst stage markedly decreases when both the time from fertilisation to first cleavage and the percentage of fragmented and degenerated embryos at the time of the first cleavage increase. Moreover, a differential influence of energy substrate on the successful development of embryos that first cleaved at different time intervals has also been observed. The blastocyst development stage and the average number of cells per blastocyst in early- and late-cleaving embryos significantly differ between embryos cultured with glucose and those cultured with pyruvate and lactate. Culture with pyruvate and lactate, but not with glucose, causes a detrimental effect on late-cleaving embryos (30-48 h pi). It increases the proportion of early blastocysts with fewer average cells.

On the other hand, a new suitable PCR-based method for sexing porcine embryos at different developmental stages with a high degree of efficiency, accuracy and specificity has been developed. This technique was designed to co-amplify two different repetitive sequences: (1) the porcine Y chromosome-specific repeat sequence *X12696*[1], and (2) the porcine multicopy 12S rRNA mitochondrial gene. The presence or absence of the former determines samples to be male or female, respectively. In contrast, the presence of the latter, common to both sexes, distinguishes amplification failures or female embryos when Y-specific product is absent. The results shows that the procedure is sensitive enough (0.001 ng DNA) to identify the sex of porcine single cells and IVP cell-stage embryos (2-, 4-, 8- and 16-cell embryos) with 100% efficiency, as well as the sex of blastocysts with 96.6% accuracy and 96.7% efficiency.

This PCR technique was used to determine sexual dimorphisms in terms of selective loss of embryos of a determined sex under the culture conditions described herein. The results show a sex ratio deviation towards males among cleavage-stage embryos that completed their first cell cycle within 26 and 30 h pi and towards females among those embryos that underwent the first cleavage later, in both glucose- or pyruvate and

[1] GenBank Accesion Number. GenBank Home – National Center for Biotechnology Information. Available on-line via <http://www.ncbi.nlm.nih.gov/genbank/>

lactate-based media. However, our findings also reveal that the energy substrate present in the medium during the first 48 h of culture has a differential and critical effect on the selective loss of embryos of one determined sex during their development up to the blastocyst stage. Particularly, the suboptimal conditions associated with glucose in the culture medium lead to a selective loss of male embryos first cleaved between 26 and 30 h pi, resulting in a similar proportion of male and female blastocysts at 168 h pi. In addition, the decrease in blastocyst formation rate in those embryos cultured with 1.0 $mg \cdot ml^{-1}$ HA seems to be caused by a preferential loss of female embryos, leading to a slight skew in the sex ratio towards males in the group of embryos cultured with pyruvate and lactate, and by an equal loss of embryos of both sexes in the glucose group.

In conclusion, all together these results provide new knowledge about physiology, development and sexual dimorphisms in survival rates and kinetics of development in porcine embryos produced under specific *in vitro* conditions. In addition, this thesis establishes new conditions that may enhance porcine IVP techniques and develops a new suitable procedure able to determine the sex of porcine embryos at different developmental stages.

Resum

La present tesi tracta alguns dels reptes que actualment afecten a la producció *in vitro* (PIV), les tècniques de determinació del sexe i les diferències de supervivència entre embrions porcins mascles i femelles PIV sota unes determinades condicions de cultiu. En producció animal, el sexe és considerat un dels trets genètics més importants. La possibilitat de seleccionar eficaçment el sexe de la descendència ha estat sempre un dels principals objectius perseguits no només pels ramaders, sinó també pels investigadors. La determinació i selecció del sexe dels embrions porcins PIV, utilitzades conjuntament amb la criopreservació i les tècniques de transferència embrionària, poden ser emprades per manipular la proporció de sexes de la descendència. La desviació de la proporció de sexes pot accelerar els programes de millora genètica, reduir l'interval generacional i, conseqüentment, incrementar l'eficiència biològica i econòmica de la productivitat. D'altra banda, la investigació sobre la pèrdua selectiva d'embrions porcins mascles o femelles sota condicions específiques de cultiu pot permetre conèixer nous marcadors fisiològics i mecanismes moleculars responsables de la desviació de la proporció de sexes. A més, pot proporcionar la base sobre com millorar l'eficiència de les tècniques de PIV d'embrions porcins, fins i tot desviant la proporció de sexes dels embrions produïts. No obstant, aquests objectius no podran ser considerats comercialment aplicables fins que les tècniques de PIV i de determinació del sexe d'embrions porcins no millorin la seva eficiència.

Amb l'objectiu de millorar les tècniques de PIV, s'han dut a terme dos estudis diferents per tal de determinar: (1) com es veu afectat el desenvolupament dels embrions porcins PIV després de substituir la glucosa del medi de cultiu durant les primeres 48 hores per piruvat i lactat; (2) com l'addició de diferents concentracions d'àcid hialurònic (AH) al medi de cultiu afecta el desenvolupament dels embrions, i si els efectes putatius de l'AH depenen del substrat energètic present en el medi; i (3) com es veu afectat el desenvolupament dels embrions PIV i cultivats amb glucosa o piruvat i lactat segons el moment de la seva primera divisió embrionària. D'altra banda, aquests dos estudis també s'han realitzat per determinar si hi ha diferències en la supervivència d'embrions mascles i femelles sota les condicions de cultiu descrites anteriorment, utilitzant una nova tècnica de PCR dissenyada amb aquesta finalitat.

En primer lloc, obviant el temps de la primera divisió embrionària i la presència d'AH en el medi de cultiu, els resultats demostren que el piruvat i el lactat a les concentracions de 0,17 i 2,73 mM, respectivament, són els substrats energètics òptims durant els primers estadis de desenvolupament dels embrions porcins. Els embrions cultivats amb piruvat i lactat presenten taxes de blastocist significativament més altes que les dels embrions cultivats amb 5,55 mM de glucosa. A més, el cultiu amb glucosa disminueix la velocitat de desenvolupament dels embrions. En segon lloc, s'ha observat que els efectes de l'AH en el desenvolupament dels embrions porcins PIV depenen de la seva concentració i no estan influenciats pel substrat energètic present al medi de cultiu durant les primeres 48 hores post inseminació (h pi). Malgrat que l'addició de 0,5 mg·ml^{-1} d'AH al medi de cultiu no té efecte en el desenvolupament dels embrions, 1,0 mg·ml^{-1} d'AH disminueix significativament les taxes de blastocist dels embrions cultivats amb ambdós substrats energètics. Finalment, s'ha demostrat que l'èxit del desenvolupament embrionari fins a l'estadi de blastocist disminueix notablement a mesura que el temps des de la fecundació fins la primera divisió embrionària, i el percentatge d'embrions fragmentats i degenerats a la primera divisió mitòtica augmenten. A més, s'ha observat que el substrat energètic té un efecte diferencial en el desenvolupament dels embrions dividits per primera vegada a diferents temps. El cultiu amb piruvat i lactat incrementa la proporció de blastocists amb estadis primerencs i amb un nombre menor de cèl·lules en aquells els embrions dividits tardanament (entre 30-48 h pi). Aquestes diferències no s'han observat en els embrions cultivats amb glucosa.

D'altra banda, s'ha desenvolupat una nova tècnica de PCR per a poder determinar el sexe d'embrions porcins en diferents estadis de desenvolupament amb una alta eficiència, especificitat i precisió. Aquesta tècnica s'ha dissenyat per amplificar conjuntament dues seqüències repetitives diferents: (1) la seqüència repetitiva *X12696*[2] del cromosoma Y de porcí, i (2) la seqüència multicòpia del gen mitocondrial 12S RNA de porcí. La presència o absència de la primera determina que les mostres pertanyin a mascles o femelles, respectivament, mentre que la presència de la segona ajuda a distingir entre problemes d'amplificació o mostres femella, quan l'amplicó de la seqüència específica del cromosoma Y no hi és present. Els resultats mostren que la tècnica és suficientment sensible (0,001 ng DNA) per determinar el sexe de cèl·lules individuals i d'embrions porcins en estadi de cèl·lules amb un 100% d'eficiència, així

[2] Veure nota de página (1)

com també el sexe de blastocists amb una precisió i eficiència del 96.6% i 96.7%, respectivament.

Aquesta tècnica de PCR ha estat utilitzada per determinar dimorfismes sexuals en relació a la pèrdua selectiva d'embrions d'un determinat sexe en les condicions de cultiu descrites anteriorment. Els resultats mostren una major proporció d'embrions mascles entre els embrions en estadi de cèl·lules que han experimentat la seva primera divisió mitòtica entre les 26 i 30 hores pi, i una major proporció d'embrions femelles entre els embrions que s'han dividit per primera vegada més tard, independentment del substrat energètic present en el medi de cultiu durant les primeres 48 hores. No obstant, s'ha demostrat que el substrat energètic té un efecte diferencial en la pèrdua selectiva d'embrions d'un determinat sexe durant el seu desenvolupament fins a l'estadi de blastocist. Concretament, les condicions subòptimes associades al cultiu amb glucosa han provocat una pèrdua selectiva d'embrions mascles dividits per primera vegada entre les 26 i 30 h pi, obtenint una proporció similar de blastocists mascles i femelles a les 168 h pi. A més, la disminució de les taxes de blastocist en aquells embrions cultivats amb 1,0 mg·ml^{-1} d'AH sembla ser provocada per una pèrdua preferencial d'embrions femella, donant lloc a una major proporció de blastocists mascles entre els embrions cultivats amb piruvat i lactat.

En conclusió, aquesta tesi ofereix nous coneixements sobre la fisiologia, el desenvolupament i els dimorfismes sexuals en les taxes de supervivència i la cinètica del desenvolupament d'embrions porcins PIV sota unes condicions específiques. A més, estableix noves condicions de cultiu necessàries per millorar les tècniques de PIV d'embrions porcins, i presenta una nova tècnica apte per a determinar el sexe d'embrions porcins a diferents estadis de desenvolupament.

Resumen

La presente tesis trata algunos de los retos que actualmente atañen a la producción *in vitro* (PIV), las técnicas de determinación del sexo y las diferencias de supervivencia entre embriones porcinos machos y hembras PIV bajo unas determinadas condiciones de cultivo. En producción animal, el sexo es considerado como uno de los factores genéticos más importantes. La posibilidad de seleccionar eficazmente el sexo de la descendencia ha sido siempre uno de los principales objetivos de los criadores de ganado, así como también de los investigadores. La determinación y selección del sexo de los embriones porcinos PIV, utilizadas conjuntamente con la criopreservación y las técnicas de transferencia embrionaria, pueden utilizarse para manipular el sexo de la descendencia. El sesgo en la proporción de sexos puede acelerar los programas de mejora genética, reducir el intervalo generacional y, por lo tanto, incrementar la eficiencia biológica y económica de la productividad. Por otro lado, la investigación sobre la pérdida selectiva de embriones porcinos machos o hembras bajo condiciones de cultivo *in vitro* específicas pueden dar a conocer nuevos marcadores fisiológicos y mecanismos moleculares responsables del sesgo en la proporción de sexos. Además, puede proporcionar la base sobre cómo mejorar la eficiencia de las técnicas de PIV de embriones porcinos, incluso sesgando la proporción de sexos de los embriones producidos. No obstante, estos objetivos no podrán ser considerados comercialmente aplicables hasta que las técnicas de PIV y de determinación del sexo de embriones porcinos no mejoren su eficiencia.

Con el objetivo de mejorar las técnicas de PIV, en la presente tesis se han realizado dos estudios diferentes para determinar: (1) cómo se ve afectado el desarrollo de los embriones porcinos PIV después de sustituir la glucosa del medio de cultivo durante las primeras 48 horas por piruvato y lactato; (2) cómo la adición de diferentes concentraciones de ácido hialurónico (AH) al medio de cultivo afecta al desarrollo de los embriones, y si los efectos potenciales del AH dependen del sustrato energético presente en el medio; y (3) cómo se ve afectado el desarrollo de los embriones PIV y cultivados con glucosa o piruvato y lactato según el momento de su primera división embrionaria. Por otro lado, estos dos estudios también se han realizado para determinar diferencias en la supervivencia de embriones machos y hembras bajo las condiciones de cultivo descritas anteriormente, utilizando una nueva técnica de PCR diseñada para este fin.

En primer lugar y sin tener en cuenta el tiempo de la primera división embrionaria y la presencia de AH en el medio de cultivo, los resultados demuestran que el piruvato y el lactato a concentraciones de 0,17 y 2,73 mM, respectivamente, son los sustratos energéticos óptimos durante los primeros estadios de desarrollo de los embriones porcinos. Los embriones cultivados con piruvato y lactato presentan tasas de blastocisto significativamente más altas que las de los embriones cultivados con 5,55 mM de glucosa. Además, el cultivo con glucosa disminuye la cinética de desarrollo de los embriones. En segundo lugar, se ha observado que los efectos del HA en el desarrollo de los embriones porcinos PIV dependen de su concentración, y no se ven influenciados por el sustrato energético presente en el medio de cultivo durante las primeras 48 horas post inseminación (h pi). Mientras que la adición de 0,5 $mg \cdot ml^{-1}$ de AH al medio de cultivo no tiene efecto sobre las tasas de desarrollo, 1,0 $mg \cdot ml^{-1}$ disminuye significativamente las tasas de blastocisto de los embriones cultivados con ambos sustratos energéticos. Finalmente, se ha demostrado que el éxito del desarrollo embrionario hasta el estadio de blastocisto disminuye notablemente a medida que el tiempo desde la fecundación hasta la primera división embrionaria, y el porcentaje de embriones fragmentados y degenerados en la primera división mitótica aumentan. Además, se ha observado que el sustrato energético tiene un efecto diferencial en el desarrollo de los embriones divididos por primera vez a distintos tiempos. El cultivo con piruvato y lactato incrementa la proporción de blastocistos en estadios tempranos y con un menor número de células en aquellos embriones divididos tardíamente (entre 30-48 h pi). Estas diferencias no se han observado en los embriones cultivados con glucosa.

Por otro lado, se ha desarrollado una nueva técnica de PCR para poder determinar el sexo de los embriones porcinos en diferentes estadios de desarrollo con una alta eficiencia, especificidad y precisión. Esta técnica se ha diseñado para amplificar conjuntamente dos secuencias repetitivas diferentes: (1) la secuencia repetitiva *X12696*[3] del cromosoma Y de porcino, y (2) la secuencia multicopia del gen mitocondrial 12S RNA de porcino. La presencia o ausencia de la primera determina que las muestras pertenezcan a machos o hembras, respectivamente, mientras que la presencia de la segunda ayuda a distinguir entre problemas de amplificación o muestras hembra, cuando el amplicón de la secuencia específica del cromosoma Y no está presente. Los resultados muestran que la técnica es suficientemente sensible (0,001 ng DNA) para determinar el sexo de células individuales y de embriones

[3] Ver nota de página (1)

porcinos en estadio de células con un 100% de eficiencia, y el sexo de blastocistos con una precisión y eficiencia del 96.6% y 96.7%, respectivamente.

Esta técnica de PCR ha sido utilizada para determinar dimorfismos sexuales relacionados con la pérdida selectiva de embriones de un determinado sexo bajo las condiciones de cultivo descritas anteriormente. Los resultados muestran una mayor proporción de embriones machos entre los embriones en estadio de células que han experimentado su primera división mitótica entre las 26 i 30 horas pi y una mayor proporción de embriones hembra entre los embriones que se han dividido por primera vez más tarde, independientemente del sustrato energético presente en el medio de cultivo durante las primeras 48 h. No obstante, se ha demostrado que el sustrato energético tiene un efecto diferencial en la pérdida selectiva de embriones de un determinado sexo durante su desarrollo hasta el estadio de blastocisto. Concretamente, las condiciones subóptimas asociadas al cultivo con glucosa han dado lugar a una pérdida selectiva de embriones machos divididos por primera vez entre las 26 y 30 h pi, obteniendo una proporción similar de blastocistos machos y hembras a las 168 h pi. Además, la disminución de las tasas de blastocisto en aquellos embriones cultivados con 1,0 $mg \cdot ml^{-1}$ de AH parece ser provocada por la pérdida preferencial de embriones hembra, sesgando la proporción de sexos a favor de los machos entre los blastocistos cultivados sólo con piruvato y lactato, y no con glucosa.

En conclusión, esta tesis ofrece nuevos conocimientos sobre la fisiología, el desarrollo y los dimorfismos sexuales relacionados con las tasas de supervivencia y cinética del desarrollo de los embriones porcinos PIV bajo condiciones específicas. Además, establece nuevas condiciones de cultivo necesarias para mejorar las técnicas de PIV de embriones porcinos, y presenta una nueva técnica apta para determinar el sexo de embriones porcinos en diferentes estadios de desarrollo.

Part I
Introduction

Chapter 1
Introduction

1.1 Thesis Outline

This thesis is about challenges that currently concern *in vitro* production (IVP), sex determination procedures and sex-related survival of *in vitro*-produced porcine embryos. It is built as an **article compendium**, preceded by this introduction, which presents the set of articles, and an extended bibliographic revision, and closed by the general discussion and conclusions derived from them. It also includes a summary and its translation into Catalan and Spanish.

In this introduction (**Chapter I**), an outline of the thesis (Section 1.1) and a general and brief state of the art (Section 1.2) are set out in order to put in context and justify the issues that this thesis addresses. In Section 1.3, the main objectives are described. **Chapter II** is an extended bibliographic revision of the three main points that concern this thesis: (1) importance of reproductive biotechnologies in swine (Section 2.1), (2) *in vitro* production of porcine embryos (Section 2.2), and (3) embryo sex determination and selection (Section 2.3). The results and the methodology of the present investigation are comprised within three main articles, and are found in Chapters III, IV and V. **Chapter III** deals with the development of a suitable PCR procedure for sexing porcine embryos at different developmental stages with a high degree of efficiency, accuracy and specificity. Conversely, **Chapter IV** and **Chapter V** study how the addition of some energy sources and glucosaminoglycans may improve *in vitro* culture (IVC) and the development of *in vitro*-produced porcine embryos. They also determine how the development of IVP porcine embryos, cultured with glucose or pyruvate and lactate, is affected by the timing of the first embryonic cleavage. Moreover, they also study whether these new IVC conditions differentially affect the survival of male and female embryos, through determining any sex ratio deviation of the resulted embryos. Finally, **Chapter VI** and **Chapter VII** deal with the most important findings obtained in the articles as a whole and summarises the global conclusions, respectively.

1.2 State of the art

Assisted reproductive technologies in domestic animals have experienced tremendous growth over recent decades, and they have developed into new techniques that present both swine industries and biomedical research with numerous opportunities. Some of these reproductive technologies are in widespread use for commercial pork operations, such as artificial insemination (AI) (Riesenbeck, 2011); however, others still need to be improved and they are in limited use in specific segments of both the industry and the research field compared to those in other species such as bovine (Knox, 2011). Examples of the latter group are IVP of porcine embryos and sex preselection of the offspring.

Sex is the individual characteristic that exerts the largest impact on mammalian phenotype (Ober *et al.*, 2008). Until recently, although it remained a matter of chance, the ability to predetermine the sex in farm animals has been one of the main goals pursued by both breeders and researchers. Sperm sexing by flow citometry and sex determination by embryo biopsy are currently the only reliable methods to obtain offspring of a determinate sex (Niemann *et al.*, 2003; Johnson *et al.*, 2005; Naqvi, 2007). However, sperm sexing by flow cytometry (Johnson and Clarke, 1988; Johnson, 1997) has severe limitations because of the small number of cells that can be sorted per hour, the high cost of the equipment and the lower fertility that has been reported, especially compared with the use of non-sorted semen samples for assisted reproductive technologies (Niemann *et al.*, 2003; Bodmer *et al.*, 2005; Frijters *et al.*, 2009; Garner *et al.*, 2013). In contrast, embryo sex determination is a potential alternative for both commercial and research purposes.

Sex preselection of porcine *in vitro* preimplantation embryos, used together with cryopreservation and embryo transfer (ET) techniques, can be used to manipulate the sex ratio of offspring. This can improve the herd for desired purposes, reduce the generation interval, accelerate genetic improvement programmes and, thus, increase the biological and economic efficiency of productivity (Jonhson *et al.*, 2005). Moreover, carrying out research on selective loss of porcine male or female embryos under specific *in vitro* conditions would highlight novel physiological markers and molecular mechanisms responsible for those sex ratio skews. In addition, it would provide the basis of how *in* vitro production can be enhanced in a sex-related manner and thus, it would offer a method for altering sex ratio of embryos produced for transfer commercially in a lesser cost and time-consuming way (Bermejo-Alvarez, 2010). Nevertheless, these goals cannot be considered commercially applicable until the

porcine IVP (**PAPER II** and **PAPER III**) and sex determination (**PAPER I**) procedures improve their efficiency.

A large source of cleavage stage embryos and blastocysts is needed for practical application of embryo sex preselection techniques. However, despite many efforts to improve IVP systems in pigs, the efficiency of this technique is still low compared to its *in vivo* production counterpart and to those in other species, such as bovine one. Up to now, development rates of IVP blastocysts range from 6-7% to 20-30%, depending on laboratories and culture conditions (Nagai *et al*., 2006; Isom *et al*., 2012). Although the main limiting factor that controls the efficiency of IVP mammalian embryos appears to be the oocyte and zygote quality (Lonergan *et al*., 1999; Booth *et al*., 2007; Isom *et al*., 2012), the composition of the culture medium and its conditions are also critical for the subsequent pre- and postimplantational embryo development (Kidson, 2004; Rubessa *et al*., 2011). Several recent studies have shown a strong correlation between oocyte competence, early zygotic cleavage and subsequent embryonic development (Dang-Nguyen *et al*., 2010; Isom *et al*., 2012). Moreover, many laboratories have been trying to supplement culture media with different energy substrates (Kikuchi *et al*., 2002; Karja *et al*., 2006), glycosaminoglycans (Kim *et al*., 2005a), hormones (Lee *et al*., 2005) or other regulatory molecules at different concentrations and timings in order to make media as similar as possible to the microenvironment found *in vivo*. It is known that the presence of optimal concentrations of specific energy substrates, for instance, at each stage of embryonic development is crucial, since it determines the optimal embryo metabolism to develop properly (Swain *et al*., 2002; Rubessa *et al*., 2011).

Concerning these notions, the study and availability of contentious results regarding how the replacement of some energy substrates with others at different stages of embryo development affects the kinetics of the first embryonic divisions, and subsequent embryo development are needed. Moreover, it is important to determine the effects of adding other substances at different concentrations, such as hyaluronic acid (HA), which is known to play an important role in reproductive physiology, and its interaction with the energy substrates present in the culture media. These questions will be addressed in **PAPER II** and **PAPER III**.

On the other hand, due to sex-related physiological, transcriptional and epigenetic differences, phenotypic dimorphism has been reported for male and female bovine preimplantation embryos under some specific culture conditions. It include differences in speed of embryo development, survival after vitrification, cell number at blastocyst stage, and metabolism (Gutiérrez-Adán *et al*., 2001; Helle *et al*., 2008; Bermejo-Alvarez *et al*., 2011). However, to our knowledge, this information is not yet available for porcine embryos (**PAPER II** and **PAPER III**).

In addition, although several approaches have been attempted to determine the sex of mammalian embryos, the low efficiency, accuracy and speed of these methods preclude their routine application in the field. In contrast, polymerase chain reaction (PCR)-based assays have undergone rapid development and their use for sexing bovine embryos has become increasingly prevalent due to their sensitivity, specificity, speed and cost-effectiveness (Shea 1999; Bredbacka 2001; Park *et al.,* 2001; Bermejo-Alvarez *et al.,* 2008a; Rattanasuk *et al.,* 2011). However, because of the few genes and sequences that, so far, have been sequenced and mapped from the porcine genome, molecular genetics in sexing techniques are poorly developed in swine (Niemann *et al*., 2003). Accordingly, few PCR-based methods for porcine embryo sex determination have been developed, and they are not sufficiently sensitive, accurate, efficient and specific to be used for porcine sex determination using small amounts of genomic DNA (Pomp *et al*., 1995; Sathasivam *et al*., 1995; Fontanesi *et al*., 2008).

Therefore, what is required by researchers when determining sex-dependent differences in porcine embryo physiology and survival under different culture conditions (**PAPER II** and **PAPER III**) is the availability of suitable procedures to allow sex determination of porcine embryos, with a high degree of efficiency and accuracy (**PAPER I**). The development of highly sensitive sex determination procedures is also really needed for the sex determination of biopsied blastomeres and the subsequent biopsied embryo culture and transfer to obtain offspring of a determined sex. In addition, identifying conditions that lead to deviation from 1:1 sex ratio would give insight into factors controlling sex ratio and would offer a method for altering sex ratio of embryos produced for transferring commercially in a lesser cost and time-consuming way.

1.3 Objectives

Based on all stated in the previous section, this thesis proposes six main objectives according to challenges that currently concern *in vitro* production, sex determination and sex-related survival of porcine embryos. Three papers have been published in response to these six objectives, which are named at the end of each aim. In concrete, the objectives are:

1. To develop a suitable PCR-based method for sexing porcine embryos at different developmental stages with a high degree of efficiency, accuracy and specificity (**PAPER I**).

2. To determine the effects on the development of IVP porcine embryos of replacing glucose with pyruvate and lactate in the culture medium for the first 48 h of culture (**PAPER II** and **PAPER III**).

3. To determine the effects on the development of IVP porcine embryos of adding different concentrations of HA to the culture medium and whether these putative effects of HA depend on the major energy substrate of the culture medium (**PAPER II**).

4. To determine whether the timing of the first embryonic cleavage affect the development of IVP porcine embryos cultured with glucose- or pyruvate- and lactate-based media (**PAPER III**).

5. To determine sexual dimorphisms in terms of selective loss of embryos of a determined sex under different conditions: a) the absence or presence of two different concentrations of HA during all the culture period, together with b) the presence of glucose or pyruvate and lactate in the IVC medium during the first 48 h of culture (**PAPER II**).

6. To determine dimorphisms in terms of selective loss of embryos of a determined sex on embryos cleaved for the first time at different time intervals, and cultured with glucose- or pyruvate- and lactate-based media during the first 48 h of culture (**PAPER III**).

Part II
Bibliographic Revision

Chapter 2

Bibliographic Revision

2.1. Importance of Reproductive Biotechnologies in Swine

2.1.1 Swine Industry for Meat Production

Around the world, meat is a valuable source of protein that helps to supplement the intake of essential nutrients for humans. The pig is efficient for conservation of a variety of available food stuffs into high quality protein. In addition, among livestock species, the pig is noted for its high fertility. The efficiency of this large animal is realised from its short interval to maturity, short gestation period, multiple offspring per pregnancy, and quick tendency to rebreed (Knox, 2014).

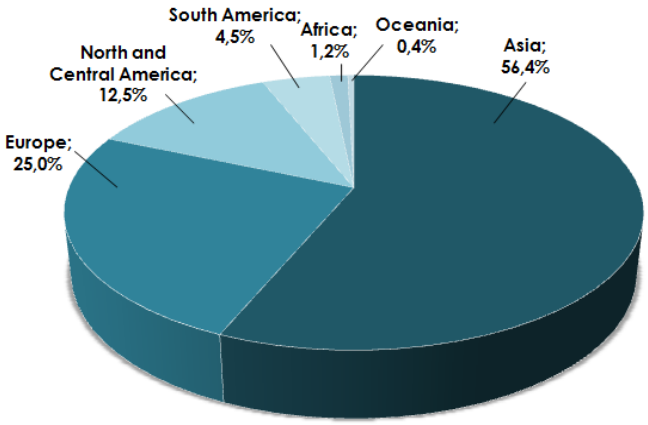


Figure 1. World production of pig meat in 2011

Modified from the *Statistics Division of the Food and Agriculture Organization of the United Nations (FAOSTAT)*. February 2013. Avaliable via: <http://faostat.fao.org/site/339/default.aspx>

Regarding these notions, pigs have been playing an important role in meat production in many countries. In 2011, pork meat accounted for 38.4% of the total meat production in the world, and the largest worldwide producers were Asia (56.4 %), Europe (25 %) and North and Central America (12.5 %; **Figure 1**).

The highest annual apparent consumption in the European Union (EU) of meat products was recorded for pork products, averaging 37 kg per capita in the EU, a value that is similar to the sum of meat from poultry, cattle, sheep and goats (European Union, 2011). The production of pig meat in the EU was over 22 million tonnes with above 145 thousands of censed animals in 2012 (European Union, 2013). Since 2001, after Germany, Spain has been the second largest pig producer in the EU, as well as the fourth worldwide pig producer beneath China, the United States and Germany (**Figure 2** and **Figure 3**).

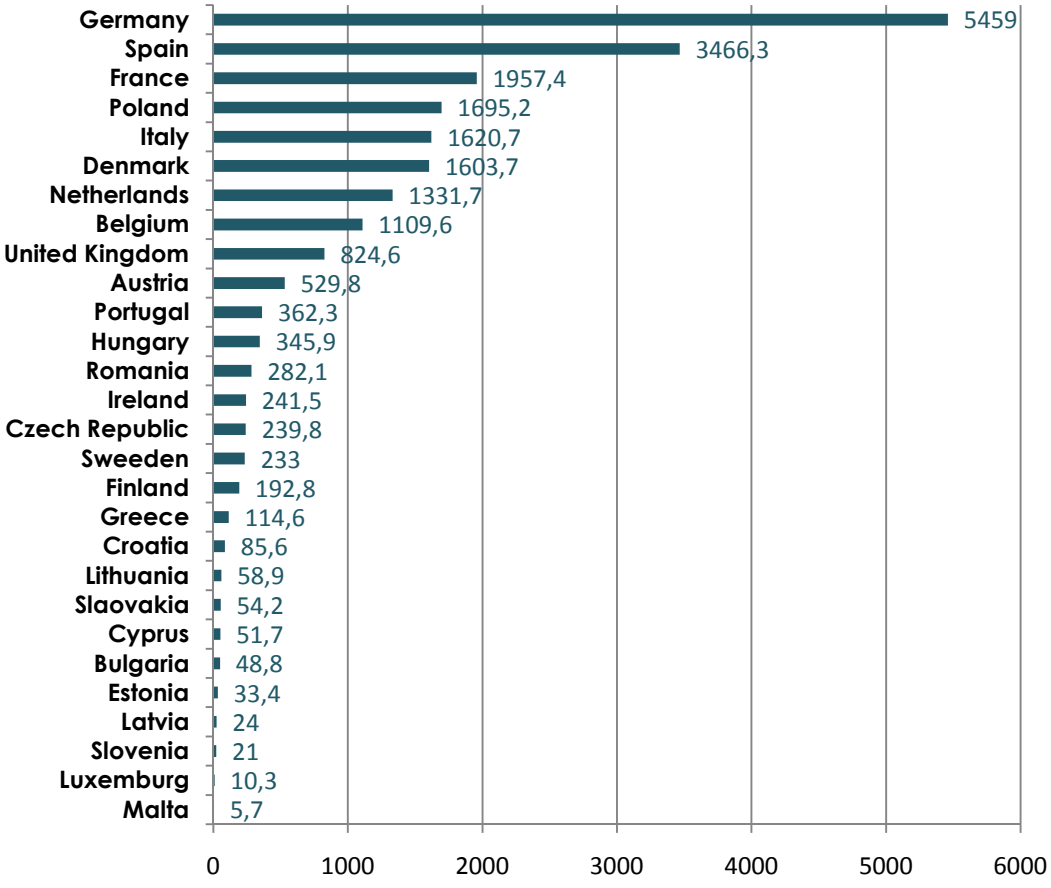


Figure 2. Production of pig meat in the EU in 2012 (1000 tonnes of carcass weight)

Modified from the *Statistical Office of the European Communities (EUROSTAT)*. 2013. *Eurostat Pocketbooks. Agriculture, forestry and fishery statistics.* Avaliable on-line via *Eurostat Home - European Comission*: <http://epp.eurostat.ec.europa.eu/portal/page/portal/product_details/publication?p_product_code=KS-FK-13-001>

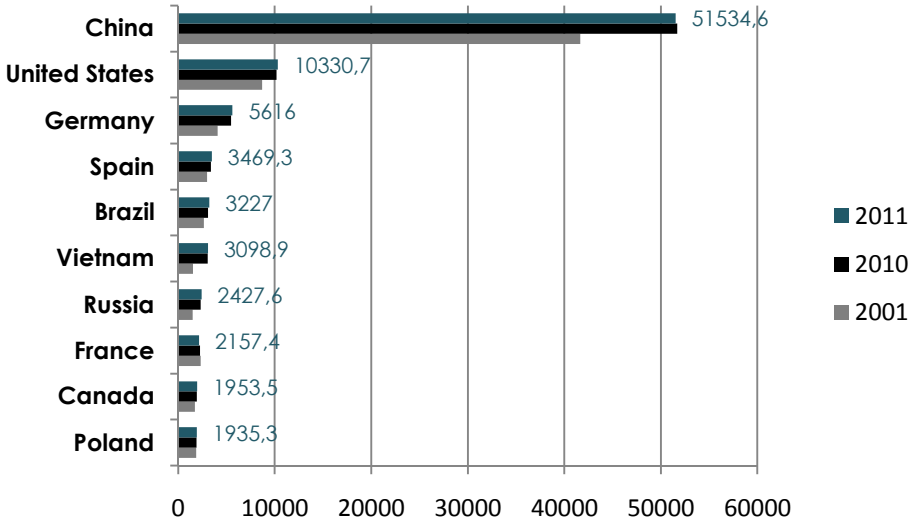


Figure 3. Ranking of the largest worldwide pig producers in 2001, 2010 and 2012

Numbers in green mean 1000 tonnes of carcass weight, the production of pig meat in 2011. Modified from the *Statistics Division of the Food and Agriculture Organization of the United Nations (FAOSTAT)*. February 2013. Avaliable on-line via: <http://faostat.fao.org/site/339/default.aspx>

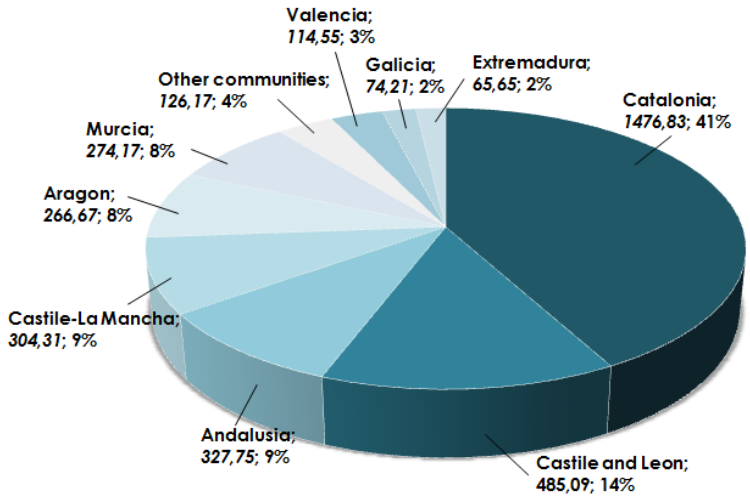


Figure 4. Production of pig meat in Spain in 2012 (*1000 tonnes of carcass weight)*

Modified from *Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural (DAAM). Generalitat de Catalunya. Juliol 2013. Informe del sector porcí. Exercici 2012.* Avaliable on-line via: <http://gsp.iws.es/sites/default/files/u6/Informe%20del%20sector%20porc%C3%AD%20catal%C3%A0%202012.pdf>

Catalonia is the first Spain community in number of pig livestock with above 6.5 million censed in 2012 (DAAM, 2013). With an annual production of more than 3.5 million tonnes of pork meat, over 1.4 million tonnes in Catalonia, pork industry in Spain is the highest livestock sector accounting for over 4000 millions of Euros per year (**Figure 4**).

Thereby, the availability of efficient pork production systems in terms of quality, number and desirable sex of the offspring born has important economic consequences, especially in those countries where pork industry is a significant source of income. For that reason, research in genetic improvement of production traits (e.g., growth rate, meat percent, feed efficiency, piglet production), but also in offspring sex ratio manipulation according to the market demand is needed with the main purpose of improving pork quality for its consumption.

2.1.2 Pig for Biomedical Purposes

Research in pigs is important not only for agricultural purposes, but also for their use as models in biomedical studies. The increasing life expectancy of humans has led to growing numbers of patients suffering from chronic diseases and end-stage organ failure. In addition, organ failure also appears in newborns and during infancy. The demand for organ transplantation already surpasses by far the number of available donors. This situation, together with the serious shortage of human organs for transplantation, results in the need for the development of xenotransplantation (Klymiuk *et al*., 2010; Flisiokowska *et al*., 2013). Pigs share many similarities with humans in body size, anatomy, diet and their physiological and pathophysiological responses. Furthermore, they offer the possibility to be raised under the highest possible hygienic standards and, owing to their high fertility and prolificacy, large numbers of offspring can be produced. Therefore, there are few additional requirements for the use of pigs to model human disease and for the use of pig organs as human organ replacement (Nieman and Rath, 2001; Nagai *et al*., 2006; Flisiokowska *et al*., 2013).

However, although the numerous advantages of porcine xenotransplantation, the most profound barrier is the immunological rejection of the organ graft (Yang and Sykes, 2007; Le Bas-Bernardet *et al*., 2008; d'Apice and Cowan, 2009). One solution for this problem is to genetically modify pigs to allow their organs not to be recognised as foreigners when transplanted into humans. Up to now, plenty of genetically modified or transgenic pig lines have been established with the aim to overcome the various rejection mechanisms of pig-to-primate xenografts (Ekser *et al*., 2009). For instance, the

identification of Gala(1,3)Gal as the major antigen target for human and non-human primate anti-pig antibodies (Good *et al*., 2002), and the introduction of techniques of nuclear and embryo transfer (Polejaeva *et al*., 2000) enable the knock-out 1,3-galactosyltransferase gene. This gene is responsible for the production of an enzyme that adds terminal Gal epitopes to many tissues in pigs (Phelps *et al*., 2003; Kolber-Simonds *et al*., 2004). However, clinical xenotransplantation will require multi-transgenic pigs transmitting effective genetic modifications in a non-segregating manner (Klymiuk *et al*., 2010). Thus, more research regarding these items is required.

Furthermore, apart from a useful model for xenotransplantation, the transgenic technology has also been applied in swine to enhance agricultural applications (growth and development), to increase disease resistance and as a useful model of human disease, as donors of embryonic stem cell lines, and as bioreactors of pharmaceutical products (Niemann and Rath, 2001; Kątska-Książkiewicz, 2006; Flisiokowska *et al*., 2013).

Given the immense potential of xenotransplantation and, despite forward steps being made in cloning and transgenesis in pigs using nuclear transfer strategies, more research in cloning and transgenesis technology is warranted to generate single- or multi-transgenic pigs as organ donors for xenotransplantation and also as pig models developed to enhance human health. Moreover, more efficient embryo production systems for pigs are required to achieve all these purposes.

2.1.3 Reproductive biotechnologies in porcine

The combined interest in swine biotechnologies by both biomedical field and swine industry creates an increased desire for the development of new technologies as well as for the implementation of the existing ones (Day, 2000). Biotechnology in livestock comprises an arsenal of reproductive biotechnological procedures and molecular genetics (Naqvi, 2007). The main objective of these procedures in pigs is to increase reproductive efficiency and rates of genetic improvement, while maintaining genetic diversity. They also offer the potential for greatly extending the multiplication and transport of genetic materials and for conserving unique genetic resources in reasonably available forms for possible future use (Okere and Nelson, 2002; Naqvi, 2007). The development and improvement of reproductive technologies are focusing on gamete and embryo collection, AI, cryopreservation of gametes and embryos, IVP of embryos, ET, and manipulation of embryos (biopsy, sexing, nuclear transfer,

production of chimeras, establishment of embryo stem cells, and gene transfer; Nieman and Rath, 2001; Okere and Nelson, 2002, Martínez *et al*., 2005).

The application of these techniques can only be understood if their research and development are done in parallel, since the final purposes for food production and biomedical research require their combination. Furthermore, reproductive biotechnologies, particularly the IVP of embryos as well as transgenic and somatic cell nuclear transfer (SCNT), are totally necessary for the research and application of molecular-genetic techniques. These include genome analysis (e.g., sequencing, mapping and determination of polymorphisms of porcine genes), molecular tests to identify genetic disorders, genetic identity and/or diversity, functional genomics (e.g., expression patterns and interaction of genes) and transgenic modification including either gain or loss of function (Nieman and Rath, 2001). Therefore, the real impact on genetic progress will come from combining new reproductive techniques with throughput of molecular techniques (Okere and Nelson, 2002, Naqvi, 2007).

The movement of genetic resources in the swine industry currently relies predominantly on the shipping of live animals. The high cost of shipping plus the risk of disease transmission are some of the disadvantages of transporting genetics via live animals (Okere and Nelson, 2002; Martinez *et al*., 2005). Moreover, apart from the problems associated with cryopreservation of boar semen (Casas and Flores, 2013), the use of AI can only change half the genetics of the offspring and may involve high risks of disease transmission (Casas, 2010; Bussalleu and Torner, 2013; Knox, 2014). Therefore, shipping embryos derived from genetic valuable boars and sows might be a more cost-effective method for disseminating genetic material (Martinez *et al*., 2005; Knox, 2014). However, the high cost of surgical collection and transfer of embryos, in conjunction with the relative inability to cryopreserve and sex porcine embryos, has limited, so far, the commercial application of this technology (Okere and Nelson, 2002). Improvements in IVP of porcine embryos, embryo cryopreservation, non-surgical embryo transfer (ns-ET) and embryo sexing procedures are required.

The development of ns-ET technology and its application in the pig industry has recently been of considerable interest. The embryo transfer includes the collection or production of embryos (*ex vivo* or *in vitro*) from donor pigs, the temporary culture and/or manipulation (such as embryo sexing) and reintroduction/transfer into a recipient animal (Okere and Nelson, 2002). The use of this technology would allow the movement of genetic resources with enhanced animal welfare, minimal risk of disease transmission and reduced transportation costs, in comparison with transport of live animals (Martinez *et al*., 2005; Knox, 2014). Furthermore, ET is also essential for the application of other reproductive biotechnologies such as embryo sex determination by embryo biopsy.

However, the commercial application of the ET in swine industry has been limited because of the requirement of surgical procedures for both the collection and transfer of the embryos and because of the difficulties for long-term storage of pig embryos (Martinez *et al*., 2005; Yoshioka *et al*., 2012). Recently, new perspectives for ET have arisen with the development of new procedures for embryo cryopreservation and ns-ET methods (Martinez *et al*., 2005; Knox, 2014).

Particularly, research efforts have been given to ns-ET and success has been achieved with improved designs in transfer instruments (Cameron *et al*., 2006), paying attention to the number and developmental age of embryos to transfer (Yoshioka *et al*., 2012) and to the volume and composition of transfer medium (Martinez *et al*., 2004; Nakazawa *et al*., 2008; Yoshioka *et al*., 2012). Moreover, variations in synchronisation of donors and recipients have been tested in order to increase ns-ET efficiency (Hazeleger and Kemp, 1999). Likewise, early research has demonstrated that the removal of part of the embryo lipid content prior to freezing using vitrification, rather than conventional freezing procedures, improves the freezability and survival rates after embryo transfer (Li *et al*., 2006b; Du *et al*., 2007; Nagashima *et al*., 2007). The development of delipidation technologies has provided evidences that intracellular lipids are linked to hypothermic sensitivity (Nagashima *et al*., 1995). In addition, vitrification has been shown to be able to elude the difficulties associated with cooling sensitivity and ice crystallisation (Cameron *et al*., 2006). Other factors that have contributed to successful cryopreservation of pig embryos at early stages include: a) the use of cytoskeletal stabilizing agents and specific cryoprotectants, b) the increase of cooling rates using open pulled straws and micro droplets, and c) the assisted hatching (Cameron *et al*., 2006).

Against this background, further development of ns-ET, long-term embryo preservation and IVP systems, together with the establishment of methods for sex determination and selection of embryos are required to provide a basis for an effective propagation of world's most valuable genetic resources for both agricultural and biomedical purposes.

2.2. *In Vitro* Production of Porcine Embryos

2.2.1. State of the Art and Applications

In vitro production of porcine embryos has been of particular interest to researchers for many years (Gil *et al*., 2010). This process includes three technological steps (**Figure 5**), which in chronological order are: (1) the *in vitro* maturation (IVM) of immature oocytes obtained from abattoir-derived ovaries, (2) *in vitro* fertilisation (IVF) of matured oocytes and (3) *in vitro* culture (IVC) of zygotes. These three steps comprise a complex set of physiological processes, each one conditioning *per se* the success or failure of the next step (Yoshioka, 2011).

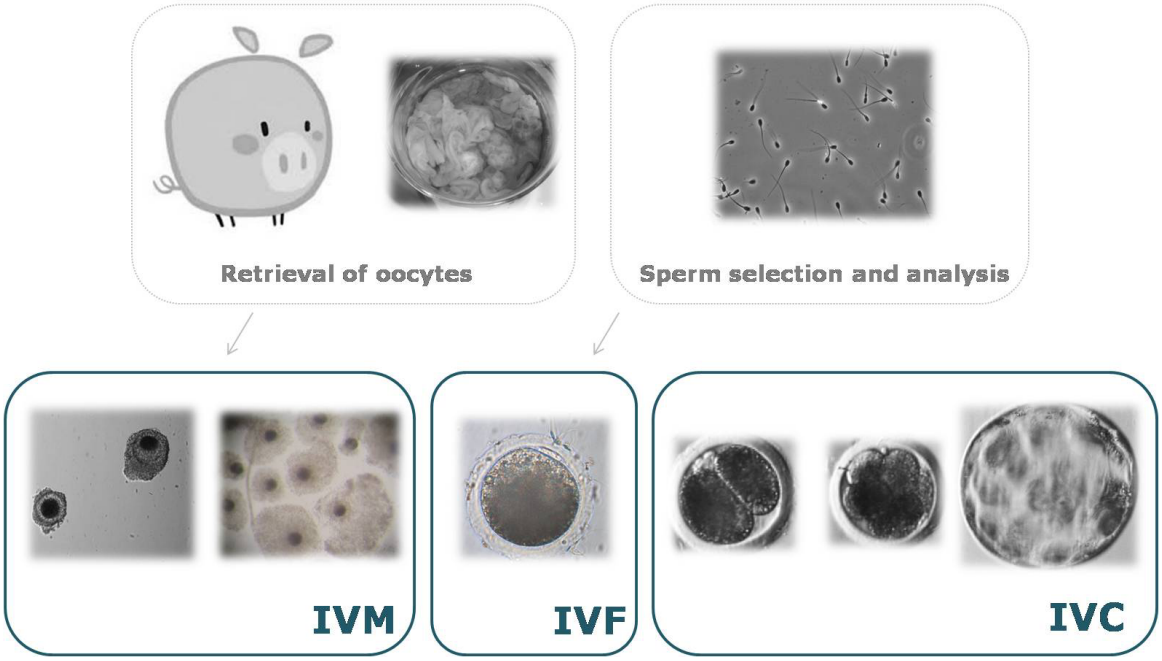


Figure 5. Schematic diagram of *in vitro* production of porcine embryos

Successful large-scale IVP of porcine embryos can now provide viable embryos more efficiently with less cost and time compared with the surgical collection of *in vivo*-derived embryos from sows. Moreover, since swine ovaries contain large numbers (>200000) of primordial follicles (Grupen *et al*., 1995), this resource provides large number of viable oocytes and, subsequently, embryos valuable not only for research in reproductive physiology, but also for the conservation of genetic resources in swine and for the production of genetically modified pigs and clones for biomedical purposes (Day, 2000; Almiñana *et al*., 2008; Yoshioka, 2011). Indeed, IVP of porcine embryos together with embryo sex determination and selection, embryo storage technology

and ns-ET can produce offspring of a predicted sex from specific superior sows and/or boars at any time and, hence, improve the dissemination of high value genetics to other farms at an affordable cost (Day, 2000; Yoshioka *et al*., 2012). Moreover, the production of a large number of embryos can benefit research in several areas, including micromanipulation of embryos to provide genetically modified swine as potential donors of tissues and organs for xenotransplantation, as well as biomodels. In addition, pork production might also benefit from the advances in IVP of porcine embryos and could gain efficiency (Day, 2000; Gil *et al*., 2010). Similarly, IVP of porcine embryos could also stimulate research on the factors controlling early embryonic development as they underlie the large economic loss that the high rate of early embryonic mortality in livestock implies (Day, 2000).

Although different IVF techniques and media available to successfully culture pig embryos for a short period had been developed previously (Iritani *et al*., 1978), the first successful production of piglets from *in vitro*-matured and *in vitro*-fertilized oocytes was reported by Mattioli *et al*. (1989), in whose experiments, 2- to 4-cell embryos at 44 h post-insemination (pi) were transferred into female recipients. However, it was not until the early 1990s that improvements in IVM, IVF and IVC systems and media have let more laboratories succeed in producing piglets following IVM and IVF from cleaved embryos at the 2- to 4-cell stage cultured for 24-36 h pi (Yoshida *et al*., 1993; Funahashi *et al*., 1996; Funahashi *et al*., 1997) and from 8-cell to morula-stage embryos cultured for 96 h pi (Abeydeera *et al*., 1998). The presence of reduced sodium chloride (NaCl) concentration or the presence of organic osmolytes in the embryo culture medium was found to provide the capability to culture single-cell embryos through the four-cell stage (Day, 2000). Notwithstanding, it was not until the last decade that porcine IVP blastocysts were demonstrated to be able to develop up to the full term when cultured for 5 or 6 days after IVF (Marchal *et al*., 2001; Kikuchi *et al*., 2002).

Although several improvements in porcine IVP techniques have been achieved and, in spite of 40000 *in vitro* pig embryos are produced each year (Terlouw and Dobrinsky, 2010), the use of IVP of porcine embryos is limited in applied production systems, as embryo production technology has not progressed far enough both in economic and technical terms (Nagai *et al*., 2006; Gajda, 2009; Dang-Nguyen *et al*., 2011; Yoshioka, 2011; Zhang *et al*., 2012). Embryos produced *in vitro* show a high rate of early developmental failure. Approximately, 10-15% of IVP embryos permanently arrest in mitosis at the 2- to 4-cell cleavage stage (Jeon *et al*., 2011). Furthermore, IVP porcine embryos are generally even less viable than both their *in vivo* produced counterparts and bovine or murine IVP embryos (Dang-Nguyen *et al*., 2011; Yoshioka, 2011). The development rates of IVP blastocysts range from 6-7% to 20-30%, depending on

laboratories and culture conditions (Nagai *et al*., 2006; Isom *et al*., 2012). Moreover, the total number of cells per blastocyst produced *in vitro* is lower than that obtained *in vivo* (58-139 vs. 150-250; Gajda and Smorag, 2004). Similarly, blastocysts produced *in vitro* are characterized by a higher number of nuclei exhibiting DNA fragmentation (Bryla *et al*., 2009) as well as more chromosomal abnormalities (MacCauley *et al*., 2003; Ulloa Ulloa *et al*., 2008). In addition, the transfer of IVP mammalian embryos results in considerable developmental abnormalities such as increased embryo mortality, extended gestation and considerably greater body weight of progeny (Gajda, 2009).

Both the high rate of early developmental failure and the low developmental competence of porcine IVP embryos can be caused by several factors. Within these factors, we can find: (1) the intrinsic characteristics of the oocytes and spermatozoa, (2) an inadequate or incomplete cytoplasmatic maturation, (3) a reduced incidence of male pronuclear formation, (4) a high incidence of polyspermy, (5) an inadequate formulation of culture media, and/or (6) suboptimal conditions for embryo culture (Abeydeera *et al*., 2002; Yoshioka, 2011).

2.2.2. Intrinsic Characteristics of the Oocyte and the Spermatozoon

The characteristics that an embryo exhibits during early development and its consequent viability following monospermic *in vitro* fertilisation are generally considered to depend largely on the quality of the oocyte, but also on the spermatozoon (Booth *et al*., 2007).

The developmental block that preimplantation embryos undergo when they are cultured *in vitro* occurs when embryonic genome takes over control of development from the maternal genome (Schoenbeck *et al*., 1992). Thus, the early cleavage divisions are mainly under maternal control, using transcripts accumulated during oogenesis. Depending on the level of specific maternal stores and the typically low gene expression profiles of most genes, early-cleaving embryos, which are extremely sensitive to their micro-environment, may signal permanent embryo arrest at this stage of development as a means to prevent further development of low-quality embryos (Betts and King, 2001). Therefore, inherent oocyte quality, which can be determined by the presence of a normal set of chromosomes and its mitochondrial genome, seems to be an important factor while determining whether an embryo will yield to premature embryonic failure or develop appropriately (Lonergan *et al*., 1999; Isom *et al*., 2012). Several recent studies have shown a strong correlation between oocyte competence and early zygotic cleavage, the latter being also conditioned by the abundance of

maternal transcripts, the number of mitochondria within the oocyte and the oocyte diameter (Lechniak *et al*., 2008).

The first zygotic cleavage, particularly the time interval from fertilisation to first cleavage, has been reported to affect the developmental competence of human (Holte *et al*., 2007), bovine (Somfai *et al*., 2010a) and porcine (Booth *et al*., 2007; Dang-Nguyen *et al*., 2010; Isom *et al*., 2012) IVP embryos. Specifically, the fastest developing embryos free of chromosomal aberrations present a higher cell number and developmental potential in bovine (Lonergan *et al*., 2000; Pers-Kamczyz *et al*., 2012) and in porcine (Dang-Nguyen *et al*., 2010; Isom *et al*., 2012) embryos. Moreover, they have fewer apoptotic cells (Vandaele *et al*., 2006; Pers-Kamczyz *et al*., 2012) and better pregnancy rates after bovine embryo transfer (Lechniak *et al*., 2008; Somfai *et al*., 2010a) compared to their slower counterparts. The better developmental ability of those embryos that cleave faster can be influenced by the number of mitochondria present in the oocyte as well as by the stability of several gene transcripts (Lonergan *et al*., 2000; Van Soom *et al*., 2007). Fast cleaved embryos show higher levels of histone 2A (H2A) and histone 3A (H3A), which are required to replace the protamines of the sperm nucleus after fertilisation and to assemble embryonic DNA into chromatin for the first cleavage. They also show higher levels of stem-loop binding protein (SLBP), which is involved in histone stabilisation, and isocitrate dehydrogenase (IDH), which modulates oxidative damage (Van Soom *et al*., 2007).

A considerable variation in the copy number of oocyte mitochondrial DNA (mtDNA) has been described both within and between species (Jansen and Burton 2004). The number of mtDNA copies varies from 10000 to 700000 in porcine oocytes and the probability of an oocyte being successfully fertilized increases with the mtDNA copy number (El Shourbagy *et al*., 2006). Moreover, the timing of the first zygotic cleavage may be related to the number of mitochondria within the oocyte, since the rate of cleaving embryos has been reported to be over 2-fold higher (46.67% vs. 22.7%) in the group of oocytes with higher number of mtDNA copies (El Shourbagy *et al*., 2006).

Another important factor contributing to oocyte competence is the oocyte diameter. Small bovine oocytes (< 110 µm) display lower cleavage and blastocyst rates, reduced blastomer number, higher incidence of apoptosis in cleaved embryos and morula, and higher DNA fragmentation before maturation than oocytes of larger diameters (> 120 µm; Vandaele *et al*., 2007). Moreover, the aberrant chromosome number is often observed in smaller (113.16 µm) rather than larger (116.83 µm) bovine oocytes (Lechniak *et al*., 2002). In addition, reaching a critical diameter of 110 µm coincides, at least in the case of bovine oocyte, with the acquisition of full competence for completing maturation up to metaphase II (Hyttel *et al*., 2001). Large oocytes that

mature early are able to process the sperm DNA more efficiently, thereby having higher probabilities to transform into more viable, early-cleaving zygotes. In addition, fast-cleaving embryos derived from large oocytes display significantly lower apoptotic cell ratio at all time points (Lechniak *et al*., 2008).

It has also been suggested that the diameter of the follicle from where the oocyte derives is strongly related to its developmental potential. Indeed, a lower developmental potential has already been demonstrated for oocytes derived from smaller follicles (< 4 mm) versus those coming from larger follicles (> 6 mm; Lequarré *et al*., 2005). The higher embryo yield of oocytes from larger follicles has been related directly to differences in messenger RNA (mRNA) transcripts of genes involved in chromatin structure, such as *H2A*, or in cell cycle regulation, such as cyclin dependent kinase subunit 1 (*CKS1B*), pituitary tumour-transforming 1 (*PTTG1*) and cyclin B2 (*CCNB2*) (Mourot *et al*., 2006). On the other hand, cumulus cells communicate each other and with the oocyte via gap junctions, which are essential for transport of nutrients and regulatory factors. Thus, a high prevalence of apoptotic cumulus cells surrounding the oocyte may predict a low developmental potential of the oocyte (Van Soom *et al*., 2007).

On the other hand, in the early years of human *in vitro* fertilisation, it was postulated that sperm quality, its contributions beyond fertilisation and early embryonic development were independent. However, today, it is known that sperm is not only a vector that delivers paternal DNA to the oocyte after fertilisation (Yeste, 2013b). Sperm also have a dynamic and critical participation in normal embryogenesis that clearly extends beyond the fertilisation process (Barroso *et al*., 2009). Moreover, suboptimal spermatozoa with damages in their DNA have been reported to negatively affect early embryo development (Ménézo, 2006; Van Soom *et al*., 2007; Yeste, 2013b).

2.2.3. *In Vitro* Culture of Embryos

Since, as stated, the main limiting factor that controls the efficiency of *in vitro* producing mammalian embryos is the oocyte and zygote quality, research should be focused on improving the quality of zygotes produced by IVF, rather than on developing maturation/culture media (Hardy *et al*., 2001). However, the IVC of embryos, which is the longest step in IVP procedure, is the period when the highest percentage of system losses takes place and when the quality of the embryos and blastocysts is determined (Rizos *et al*., 2002). The reasons by which many efforts have been used in improving maturation/culture media are not only related with the yield of embryos for transfer but they also look for minimizing the effects of stress that may

impair further foetal development (Summers and Biggers, 2003). Therefore, the composition of the culture medium and its conditions are critical for the subsequent embryo development (Lane and Gardner, 2007; Dang-Nguyen *et al*., 2011).

In vitro culture per-se and components of IVC medium have been proven to influence, even in the absence of visible effects, the pre- and postimplantacional embryonic development of mammalian embryos. It is known that culture media may affect the mammalian embryo cryotolerance (Men *et al*., 2005; Castillo-Martín *et al*., 2013a) and developmental potential (Karja *et al*., 2006; Wang *et al*.,2009), their ultrastructural morphology (Rizos *et al*., 2002), transcript abundance (Rizos *et al*., 2002; Lonergan *et al*., 2003; Bermejo-Alvarez 2011), incidence of chromosome abnormalities (Lonergan *et al*., 2004; Ulloa Ulloa *et al*., 2008), and pregnancy rates (Lazzari *et al*., 2002; Bermejo-Alvarez *et al*., 2012). Moreover, under certain conditions, the speed of development (Dang-Nguyen *et al*., 2010; Isom *et al*., 2012; Li *et al*., 2013) and the sex ratio (Gutiérrez-Adán *et al*., 2001; Bermejo-Alvarez *et al*., 2012) can also be altered.

In the last decade, there has been a substantial increase in knowledge regarding the physiology, biochemistry, genetics and epigenetic control of the mammalian embryo, including several studies performed on the porcine embryo (Lane and Gardner, 2007). All these advances have led to improve the formulation/composition of culture media and the type and conditions of culture systems (single-step, sequential or co-culture system) in order to mimic the microenvironment found *in vivo* and, so on, enhance the efficiency of porcine IVP in terms of developmental rates.

2.2.3.1. Key Components of Culture Media

Since the establishment of technologies for IVP of porcine embryos worldwide, a variety of media have been used for IVC of porcine embryos (**Table 1**) including modified Whitten's medium (mWM; Beckmann and Day, 1993), North Carolina State University (NCSU) 23 or 37 medium (Petters and Wells, 1993), Beltsville Embryo Culture Medium (BECM)-3 (Dobrinsky *et al*., 1996) and, more recently, Porcine Zygote Medium (PZM; Yoshioka *et al*., 2002). Although all of them support porcine embryo development to the blastocyst stage, NCSU-23 medium is now widely used followed by PZM.

The formulation of the inorganic and energy substrates of PZM medium (**Table 1**) is based on the reported concentrations of pig oviductal fluid (**Table 2**) with the addition of glutamine, hypotaurine and premixed solutions of basal medium Eagle's (BME) essential amino acids and minimum essential medium (MEM) non-essential amino acids (Yoshioka, 2011). In contrast, NCSU-23 is designed to mimic the metabolism and nutrient needs of porcine embryos containing glucose and glutamine as main energy substrates

as well as taurine and hypotaurine, among other components (Wang *et al.*, 2009). Whereas the NCSU-23 is the most commonly used medium, some authors (Im *et al.*, 2004; Yoshioka *et al.*, 2008; Wang *et al.*, 2009) reported that PZM was more effective in supporting the development of *in vitro*-matured and *in vitro*-fertilized porcine embryos when compared to NCSU-23, NCSU-37 or BECM-3 media (Im *et al.*, 2004; Yoshioka *et al.*, 2008). Nevertheless, none of them is still entirely appropriate since all are suboptimal for embryonic development compared to embryos cultured *in vivo* (Gil *et al.*, 2010).

Table 1. Composition (mM) of the most commonly used media for IVC culture of porcine embryos (modified from *Beckman and Day, 1993[1]; Dobrinsky et al., 1996[2]; Petters and Wells, 1993[3] and Yoshioka et al., 2002[4]*)

Component (mM)	mWM[1]	BECM-3[2]	NCSU-37[3]	NCSU-23[3]	PZM[4]
NaCl	68.49	94.59	108.73	108.73	108.00
KCl	4.78	6.00	4.78	4.78	10.00
$CaCl_2 \cdot 2H_2O$	-	1.71	1.70	1.70	-
$MgCl_2 \cdot 6H_2O$	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	1.19	1.19	1.19	1.19	0.40
$NaHCO_3$	25.07	25.07	25.07	25.07	25.07
NaH_2PO_4	-	-	-	-	-
KH_2PO_4	1.19	-	1.19	1.19	0.35
Glucose	5.56	5.56	5.55	5.55	-
Na-lactate	25.20	23.00	-	-	-
Na-pyruvate	0.33	0.33	-	-	0.20
Ca-lactate	1.71	-	-	-	-
Ca-(lactate)$_2 \cdot 5H_2O$	-	-	-	-	2.00
L-glutamine	-	1.00	1.00	1.00	1.00
D-Sorbitol	-	-	12.00	-	-
Taurine	-	-	-	7.00	-
Hypotaurine	-	-	-	5.00	5.00
BME amino acids (100x)	-	20.00 ml/l	-	-	20.00 ml/l
MEM amino acids (100x)	-	10.00 ml/l	-	-	10.00 ml/l
Phenol red	-	0.001 g/l	-	-	-
Gentamicin	-	-	-	-	0.05 mg/ml
Penicillin	-	-	0.18	0.18	-
Streptomycin sulphate	-	-	39 UI/ml	39 UI/ml	-

Generally, the basic formulation of culture media do not contain protein sources but they tend to be supplemented by different concentrations of fraction-V BSA, FAF-BSA, FBS or PVA depending on the media and laboratories.

Several studies have focused on the individual components present in the majority of these porcine embryo culture media. They all agree that the basic formulation of the IVC media contains: (1) simple minerals at low concentrations, (2) energy sources, (3) amino acids, (4) pH stabilizer, (5) trace elements, and (6) antibiotics (Kim *et al.*, 2007). However, many laboratories have also tried to supplement these media with different

energy sources (Gandhi *et al*., 2001; Kikuchi *et al*., 2002; Karja *et al*., 2006), amino acids (Beebe *et al*., 2009), growth factors (Makarevich *et al*., 2006), antioxidants (Lee *et al*., 2004; Jang *et al*., 2005), and/or macromolecules (Kim *et al*., 2004; 2005a) at different concentrations. As such, improvements require the inclusion of key constituents, but also the choice of proper concentrations of these constituents is critical to support growth and development. Most importantly, this involves the design of media that minimises the stressful effects that are inevitably inflicted on preimplantation embryos when placed in a chemically-defined environment (Summers and Biggers, 2003).

2.2.3.1.1. Energy Sources

Embryo metabolism is a valuable tool for evaluating and enhancing the efficiency of IVP systems since it is an indicator of embryo viability (Lane and Gardner, 1996; Gandhi *et al*., 2001; Leese *et al*., 2008). Understanding the metabolic needs of preimplantational embryos is vital for an appropriate formulation of culture medium, as well as for optimizing embryo growth and development (Swain *et al*., 2002; Leese *et al*., 2008).

Table 2. Composition of inorganic and energy substrates in porcine oviductal fluid
(modified from *Iritani et al., 1974* and *Nichol et al., 1992*)

Component (mM)	Oviductal fluid
Na^+	136.90
Cl^-	107.60
Ca^{2+}	2.64
K^+	12.40
Mg_2^+	0.25
H_3PO_3	1.09
Glucose	0.59
Pyruvate	0.21
Lactate	5.71

An excessive availability of nutrients may perturb metabolic homeostasis (Leese *et al*., 2008), resulting in either embryo mortality (Dumollard *et al*., 2009) or epigenetic alterations leading to reduced implantation, foetal malformations, and long-term health consequences in the mammalian offspring (Wyman *et al*., 2008). Indeed, if the necessary energy substrates are not present in sufficient concentrations or at the appropriate time, the embryo will be unable to develop (Swain *et al*., 2002). The main energy sources of an IVC medium are glucose, pyruvate and lactate and their

concentrations in the porcine oviduct have been reported to be 0.59 mM, 0.21 mM and 5.71 mM, respectively (**Table 2**). These values differ greatly from those present in the most commonly used medium, NCSU-23 (5.5 mM glucose, no pyruvate, no lactate; Petters and Wells, 1993), whereas they are a bit more similar to those present in PZM medium (0.2 mM pyruvate, 2.0 mM lactate and no glucose; Yoshioka *et al.*, 2002). However, it has been reported contentious results about which of those media outperforms more efficiently the porcine embryo culture in terms of blastocyst development and quality (Yoshioka *et al.*, 2008; Wang *et al.*, 2009). These findings indicate that embryos appear to be quite adaptable to the variable concentrations of energy substrates they are exposed to during *in vitro* culture. Nevertheless, it is increasingly recognised that an embryo with altered metabolism, particularly in its pre-compactation stage, results in a loss in viability (Lane and Gardner, 2007).

Table 3. Differences in the physiology of the mammalian embryo for development from the zygote to the blastocyst stage (modified from *Lane and Gardner, 2007*)

Pre-compactation stage	Post-compactation stage
Low biosynthetic activity	High biosynthetic activity
Low metabolic quotient	High metabolic quotient
Pyruvate-based metabolism	Glucose-based metabolism
Maternal genome	Embryonic genome
Single cell	Transporting epithelium
Low ability to maintain cellular homeostasis	Complex systems for maintenance of cellular homeostasis
Totipotent	Differentiation into inner cell mass and trophectoderm

The morphological changes occurring during development from zygotes to the blastocyst stage take place concomitantly with dynamic changes in the homeostasis, gene expression and metabolism of the embryo (**Table 3**). Both the declining gradient of pyruvate and lactate and the increasing gradient of glucose from the mammalian oviduct to the uterus reflect the changing nutrient preference of the developing embryo, as it switches from a carboxylic-acid-based metabolism to a glucose-based metabolism (Lane and Gardner, 2007). Thus, while preimplantation development progresses, glycolysis metabolism and glucose usage increases, and pyruvate and lactate usage decreases (Gandhi *et al.*, 2001). Particularly, at the earliest stages of development, the embryo has a low metabolic and biosynthetic activity. It predominantly uses the carboxylic acids pyruvate and lactate as its preferred energy

substrates and it is completely reliant on mitochondrial-based metabolism for ATP generation (Lane and Gardner, 2007). With the onset of embryo genome activation and the concomitant increase in biosynthesis, there is a switch in nutrient preference to a glucose-based metabolism, so that, during compactation and blastulation, glucose becomes the preferred nutrient and the production of ATP through glycolysis increases (**Figure 6**; Flood and Wiebold, 1988; Thompson *et al*., 1991; Rieger *et al*., 1992).

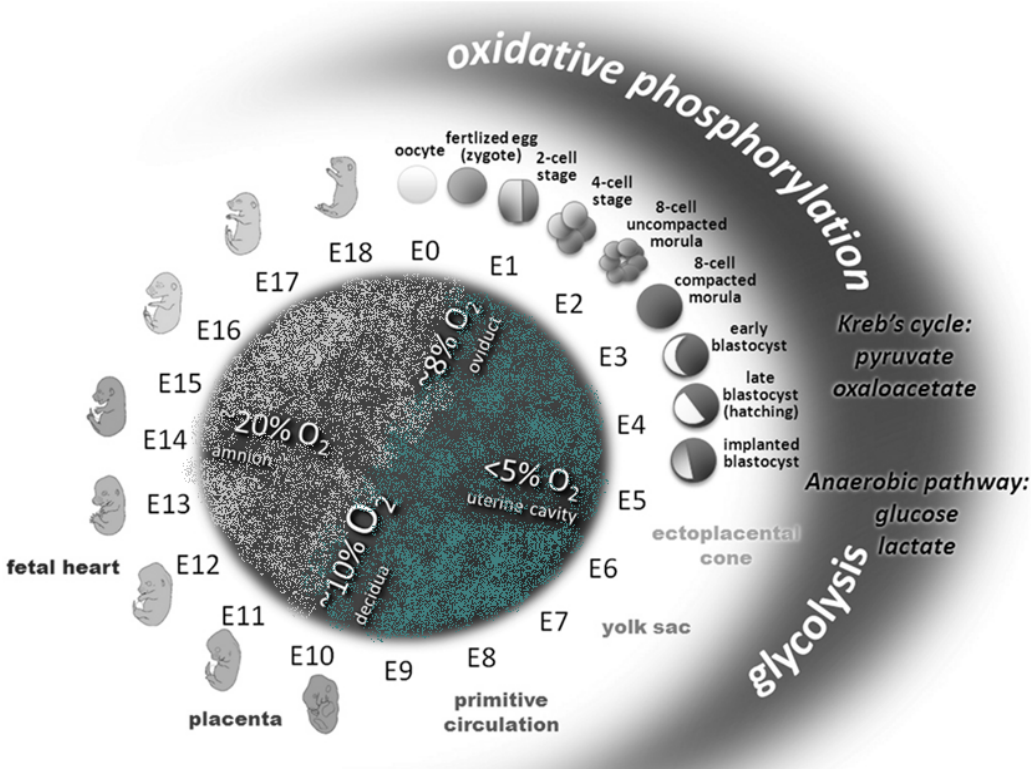


Figure 6. Oxygen and metabolic status during embryo development in mouse

Early embryos transit from relative aerobic (grey area, ~8% O2) and low glucose to anaerobic (green area, < 5% O2) and high glucose conditions. Redox metabolism transits from oxidative phosphorylation (low glucose, high oxygen) to glycolysis (high glucose, low oxygen). Pyruvate or oxaloacetate are essential substrates during early cleavage whereas glucose and lactate prevail from the eight-cell stage embryos to peri-implanted blastocyst. Modified from *Ufer and Wang, 2011*.

Some studies have reported an inhibitory role of high concentrations of glucose in the development of mouse (Chatot *et al*., 1990; Fraser *et al*., 2007; Pantaleon *et al*., 2010) and bovine (Kim *et al*., 1993; Kimura *et al*., 2005) embryos when it is present in culture before the maternal-zygotic transition, and in the development of all stages of hamster embryos (Schini and Bavister, 1988; Seshagiri and Bavister, 1989; Barnett *et al*., 1997). Murine developing embryos prefer pyruvate as their main energy source until they reach the blastocyst stage (Leese and Barton, 1984), whereas lactate is essential for the development of hamster and bovine embryos (Sturmey and Leese, 2003). Although

species-specific preferences are evident, metabolism of pig embryos is different from that of other species, as they metabolize glucose and produce lactate throughout preimplantation development (Gandhi *et al*., 2001; Swain *et al*., 2002). An additional factor that sets preimplantation porcine embryos is the abundance of small aggregates of mitochondria intimately associated with intracellular lipid vesicles; in other species, in contrast, the mitochondria are homogeneously distributed throughout the cytoplasm (Smith and Alcivar 1993). This suggests that mitochondrial metabolism is different in porcine embryos and, thus, medium requirements should be also different (Gandhi *et al*., 2001). **Table 4** shows other differences and similarities between embryos of some mammalian species.

Table 4. Differences and similarities between embryos of some mammalian species (modified from *Vajta et al., 2010*)

Characteristic	Pigs	Cattle and sheep	Mice	Humans
Volume of the zygote, metabolic reserves	Similar to humans	Similar to humans	5-8 times smaller than human	-
Cytoplasmatic lipid content	Extremely high	High	Low	Moderate
Development to blastocyst	On day 5-6	On day 6-8	On day 4-5	On day 5-6
Embryo genome activation	4-8 cell stage	8-16 cell stage	2.cell stage	4-8 cell stage
Amino acid metabolism	Similar to humans	Different from humans	Different from humans	.
Pyruvate/lactate versus glucose	No absolute need for glucose before hatching	No absolute need for glucose before hatching	Switches to glucose at 48 h	No absolute need for glucose before hatching
Overall sensitivity *in vitro*	Extremely high	High	Low	High
Genome sequencing	Almost completed	Proceeding	Completed	Complete
Genome structure	Close to humans	Unknown	Far from humans	-
Demethylation and methylation during early embryo development	Moderate	Considerable (cattle), moderate (sheep)	Extensive	Probably moderate
Time and location of embryo transfer	Flexible	Exact match required	Exact mach required	Flexible
Development anomalies after *in vitro* culture	Very rare	Frequent and serious	May occur	Very rare

At early stages of development, the porcine embryo also takes up low levels of glucose, which is predominantly metabolized through hexosamine biosynthesis and pentose phosphate pathways (PPP) (Lane and Gardner, 2007; **Figure 7**). Although metabolism of glucose through the PPP does not generate ATP, it is essential for setting the subsequent metabolic program for the maintenance of reducing power in the cell, particularly in the production of reduced glutathione for the protection against oxidative stress, and for nucleic acid and lipid biosynthesis (Swain *et al*., 2002; Lane and Gardner, 2007). In addition, the metabolism of glucose through the hexosamine biosynthesis pathway in the embryo is important as it establishes O-linked glycosylation for later stage of development (Lane and Gardner, 2007). Likewise, at the blastocyst stage, glucose is metabolized by both the tricarboxylic acid (TCA)/Krebs cycle and by aerobic glycolysis, approximately 30-50% of all of this glucose being converted to lactate. This unusual conversion of glucose to lactate in the presence of significant levels of oxygen is thought to result either from the high biosynthetic demand, or from an inability to maintain redox control in the cell (Lane and Gardner, 2007).

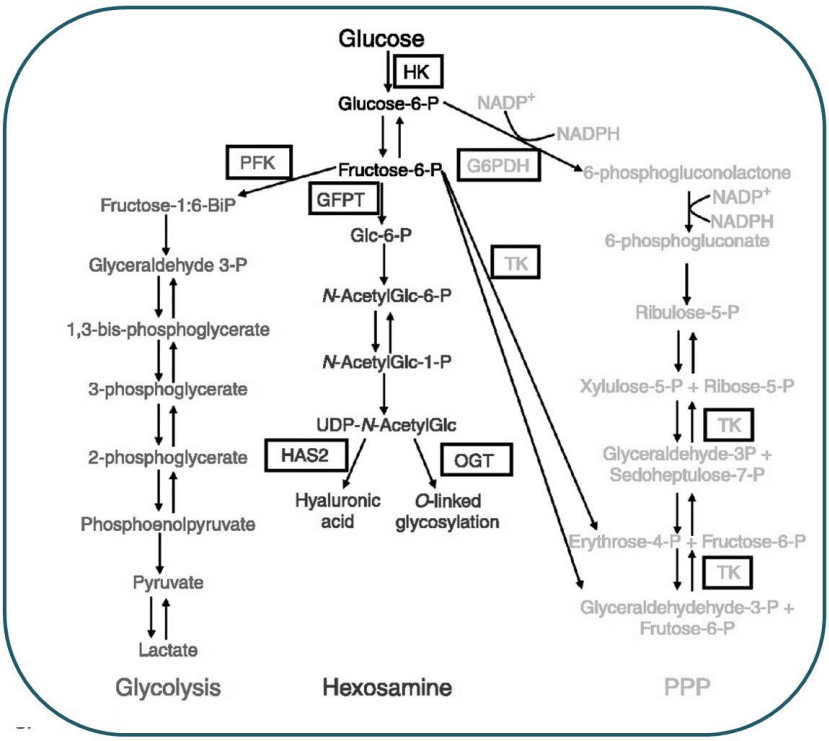


Figure 7. Metabolic pathways through which glucose can be utilised within the embryo

Pathways that are known to be active include the glycolysis (left), hexosamine pathway (middle) and pentose phosphate pathway (right). Text within boxes indicates rate-limiting or important enzymes. G6PDH, glucose-6-phosphate dehydrogenase; GFPT, glucosamine:fructose acetyl transferase; HAS2, hyaluronan synthase 2; HK, hexokinase; OGT, O-linked glycosylation transferase; PFK, phosphofructokinase; TK, transketolase. Modified from *Sutton-McDowall et al., 2010*.

On the other hand, attempts to produce pig embryos in a lower glucose concentration than that of NCSU-23 medium, such those in potassium simplex optimisation medium (KSOM) and G1.1/2.1 medium, have failed to give rates of blastocyst development comparable to those achieved with NCSU-23 medium (Machaty *et al*., 1998; Gandhi *et al*., 2001). Moreover, some studies have reported that glucose and glutamine, which are the only energy sources present in the most widely used medium (NCSU-23), can serve as the sole energy substrates for embryo development from zygote to the blastocyst stage in pigs (Petters *et al*., 1990; Swain *et al*., 2002). Therefore, glucose-containing media are commonly used for producing porcine embryos *in vitro* (Petters and Wells, 1993; Gandhi *et al*., 2001). However, the metabolic profiles of IVP porcine embryos cultured in glucose-containing medium seem not to be reflective of the correct metabolism of the *in vivo* porcine embryo. As stated previously, the concentration of glucose commonly used in the culture medium is much higher than that found in the oviduct (Nichol *et al*., 1992). Furthermore, the metabolism of *in vivo*-derived embryos differs significantly from that of *in vitro*-derived embryos, and that *in vivo*-derived embryos have higher rates of glycolysis at all development stages (Swain *et al*., 2002; Sturmey and Leese, 2003). In addition, *in vitro*-derived embryos increase their glycolytic activity after the 8-cell stage and increase lactate production, while glycolytic activity decreases for *in vivo*-derived ones (Swain *et al*., 2002).

It has been also hypothesised that metabolic activity of pig embryos, which is reflected by glucose and pyruvate uptake, as well as lactate production, differs depending on the culture medium used, since a series of interactions between components commonly present in the culture media have been described (Gandhi *et al*., 2001; Karja *et al*., 2006). In this regard, previous studies indicated an inhibitory effect of glucose on pig embryonic development (Youngs and McGinnis, 1990; Misener *et al*., 1991). However, later studies suggested that this apparent toxicity of glucose appears to be manifested only when it is present in a medium, typically lacking of amino acids, in combination with phosphate (Petters *et al*., 1990) or lactate (Abeydeera *et al*., 1999).

It has been reported that glucose, in combination with inorganic phosphate, inhibits the development of early porcine (Petters *et al*., 1990) and hamster (Seshagiri and Bavister, 1991) embryos. Embryos cultured in the presence of high levels of glucose and phosphate have their respiratory capacity and mitochondrial function reduced. Specifically, phosphate leads to an increase in glycolysis pathway rather than oxidative phosphorylation, causing an inhibitory effect on mitochondrial respiration and leading to in a loss in ATP production and, therefore, developmental arrest (Seshagiri and Bavister, 1991). Several studies have determined that this inhibitory effect of glucose in the presence of phosphate could be alleviated by adding amino acids,

ethylenediaminetetraacetic acid (EDTA) or vitamins to the culture media (Gardner and Lane, 1996; Lane and Gardner, 1998). All these components would help to prevent the loss in respiration and metabolic control and, thus, embryos would be able to maintain a more normal metabolism and adequate ATP production for growth and development.

On the other hand, although lactate is known to enhance the embryotrophic role of pyruvate, to regulate intracellular pH, the proper NAD^+:NADH ratio and the redox equilibrium in the embryo (Iwasaki *et al.*, 1999; Swain *et al.*, 2002), the combination of lactate and glucose in the same culture medium seems to be detrimental for the development of cleavage-stage pig IVP embryos (Abeydeera *et al*., 1999). Abeydeera *et al*. (1999) reported that whereas blastocyst rates achieved when embryos were cultured in NCSU-23 medium without lactate were higher than those achieved in mWM medium containing 25 mM sodium lactate (30% vs. 5%), a significant reduction in blastocyst rates was observed when embryos were cultured in NCSU-23 medium supplemented with 25 mM lactate (14% vs. 30%). In addition, the same authors observed that higher blastocyst rates were obtained when embryos were cultured with Iowa State University medium than with mWM (14% vs. 5%). Unlike mWM, the Iowa State University medium has lower levels of lactate (12.9 vs. 25 mM) and no glucose.

The first 48 h pi seem to be crucial for the embryonic development. The activation of the embryonic genome occurs between the four- to eight-cell stage, and a four-cell block is observed in pigs at this time (Jarrell *et al*., 1991; Schoenbeck *et al*., 1992). Since pyruvate and lactate are the preferred energy substrates during the early *in vitro* development of human (Hardy *et al*., 1989), mouse (Biggers *et al*., 1967; Leese and Barton, 1984) and bovine embryos (Rieger *et al*., 1992; Kim *et al*., 1993), some laboratories have tried to establish an improved IVC system for porcine embryos by replacing glucose with pyruvate and lactate in NCSU medium. However, the results obtained are inconsistent. Indeed, some studies have reported improvements in blastocyst development (Kikuchi *et al.*, 2002; Karja *et al.*, 2004) and quality of porcine IVP embryos in terms of total cell number (Kikuchi *et al.*, 2002; Beebe *et al.*, 2007) following the replacement of glucose with pyruvate and lactate, whereas others have observed no differences in blastocyst formation rate (Kim *et al.*, 2004; Beebe *et al.*, 2007; Castillo-Martín *et al*., 2013a) or cell number (Karja *et al.*, 2004; Kim *et al.*, 2004; Castillo-Martín *et al*., 2013a). However, according to these results, it has been suggested that, as in other species, pyruvate and lactate could be more optimal energy sources than glucose during early stages of porcine embryo development, but only when they are present at specific concentrations in the culture medium. It is known that correct concentrations and an optimal ratio of pyruvate and lactate are critical for regulating

the oxidation-reduction equilibrium between the cytoplasm and mitochondria, and for avoiding their detrimental effect on embryo development (Karja *et al*., 2006).

2.2.3.1.2. Protein Sources: Macromolecules

Several substrates have been used as protein source in the culture media. The most widely used for ages are foetal bovine serum (FBS) and bovine serum albumin (BSA; Dobrinsky *et al*., 1996; Wang *et al*., 1997; Kim *et al*., 2007; Zhang *et al*., 2012).

The serum is the liquid fraction resulting from the process of blood coagulation and it provides energy substrates, amino acids, vitamins, growth factors and heavy-metal chelators needed for embryo development *in vitro* (Mucci *et al*., 2006; Kim *et al*., 2007). However, the presence or concentrations of these components may vary among batches, since it depends on the sample and/or the animal from where they have been extracted. The globular un-glycosylated protein BSA is a major component of foetal bovine serum. It has an acidic character, is water soluble and has a molecular weight of about 65,000 kDa. It plays an important role in stabilizing extracellular fluid volume, since it sequesters ions and small molecules, acts as a chelator for heavy metals and other toxins, and also serves as a protein carrier for steroids, fatty acids and thyroid hormones (García-Vázquez and Matás, 2010). In addition, serum albumin is the most abundant protein in the oviductal fluid of mammals (Leese, 1988).

The effects on the embryo that justify the use of FBS and BSA in IVC are: (1) defensive effects against toxic materials within the culture media, (2) provision of substances needed for IVP of embryos, and (3) reduction of the surface tension of the medium, thereby the embryo being prevented to adhere to culture plates, pipettes and tubes (Mucci *et al*., 2006; Kim *et al*., 2007).

Both FBS and BSA have a similar role as a protein supplement. However, the possible presence of unidentifiable elements in its composition makes difficult to determine which ingredients of the serum are influential, since some aspects of its function are not yet clearly understood (Mucci *et al*., 2006). In recent years, the serum has been studied extensively to determine whether its addition to culture media definitely enhances embryo IVP efficiency in terms of quantity and quality of IVP embryos and blastocysts. The results of these studies are highly variable and even contradictory, possibly due to the variations among batches of serum in both type and concentration of embryotrophic factors. In addition, the response of embryos to the addition of FBS or BSA to culture medium, whilst not completely understood, depends on species and stage of embryos development (Gajda *et al*., 2009; García-Vázquez and Matás, 2010).

A beneficial effect of both BSA and FBS addition on the development of embryos (especially at more advanced stages of development) has been reported in pigs (Robl and Davis, 1981; Rho and Hwang, 2002; Okada *et al*., 2006). Pig blastocysts cultured with BSA or FBS have a significantly lower number of nuclei displaying DNA fragmentation and show a lower apoptotic index compared to blastocysts cultured without protein addition (Gajda *et al*., 2008). Studies conducted by other researchers have also demonstrated that BSA supplementation positively affects the first divisions of hamster embryos (McKierman and Bavister, 1992), the *in vitro* development of pig and bovine embryos (Bavister, 1995; Kircher *et al*., 1999), the formation of pig blastocysts (Rho and Hwang, 2002) and the number of cells in bovine embryos (Kircher *et al*., 1999). In addition, although some authors prefer a serum supplementation (Wang *et al*., 1997; Rizos *et al*., 2003), bovine embryos during early development are most often grown in culture medium with BSA (Bavister, 1995). Likewise, Gajda *et al*. (2008) reported that BSA supplementation significantly increased the proportion of obtained pig blastocysts compared to both FBS-containing media and protein-free culture.

Supplementing culture media with BSA appears to have a biphasic effect, because such addition inhibits the development of porcine blastocysts at early stages of growth, but also promotes development and rates of hatched blastocysts at later stages of preimplantation development (Robl and Davis, 1981; Dobrinsky *et al*., 1996; Wang *et al*., 1997). Supplementation with FBS at 20 and 48 h pi reduces blastocyst formation rates, but increases these rates when added at 96 h pi (Yoshioka *et al*., 2011). Bavister (1995) observed a similar effect for early bovine embryos. These observations have led some authors to propose a two-stage system for porcine embryo culture in which BSA is replaced with FBS when the morula develops into blastocyst (Dobrinsky *et al*., 1996; Kim *et al*., 2004).

Nevertheless, the difficulty to standardize culture conditions with the use of animal protein in the form of serum or BSA, as well as the need of avoiding their putative effects as inhibitors of embryonic development at same developmental stages have caused a growing interest in the development of media with a strictly defined chemical composition. A chemically defined medium is useful for a precise analysis of the physical action of new added substances, since it is free from unknown biological activities which may affect the embryo response. The use of a chemically defined medium also improves the reliability of media formulations, yields a higher reproducibility of results and ensures biosafety of culture media (Summers and Biggers, 2003; Yoshicka, 2011).

For these reasons, it has been suggested that the use of BSA-fraction V (BSA-V) should be replaced with the use of fatty acid-free BSA (FAF-BSA), since its extraction processes

may partially or totally eliminate the presence of undefined substances such as steroids, vitamins, fatty acids and cholesterol and, therefore, contribute to a greater product definition (Maurer, 1992; Dobrinsky *et al*., 1996).

Another attempt to use media with defined chemical composition is the replacement of both FBS and BSA by synthetic polymers. Among the most studied polymers, there are polyvinylalcohol (PVA) and polyvinylpyrolidone (PVP). Both are recognised for their role as simpler and consistent stimulus-inducing materials and their good surfactant activity, thereby avoiding adhesions gamete-gamete and/or gamete-contact surface, as well as facilitating the embryo manipulation (García-Vázquez and Matás, 2010). However, some authors have found that embryos cultured in PVA-containing media have important metabolic differences compared to those cultured in BSA-based media (Thompson and Peterson, 2000; Orsi and Leese, 2004), as well as an altered RNA transcription of relevant genes involved in embryo development (Wrenzcki *et al*., 1999).

2.2.3.1.3 Protein Sources: Amino Acids

Although the amino acid concentration in the reproductive tract can vary across species, oviductal and uterine fluids of all mammals contain significant levels of free amino acids. Moreover, oocytes and embryos possess specific transport systems for amino acids to maintain the endogenous pool (Miller and Schultz 1987; Van Winkle 2001). Likewise, the utilisation of amino acids by the embryo is flexible (Gardner, 1998), and embryos can produce amino acids in a dependent manner upon the stage of development (Kątska-Książkiewicz, 2006). Therefore, these changes in the amino acid uptake and metabolism suggest that their addition to culture medium at appropriate concentrations should be beneficial for the development of mammalian embryos (Li *et al*., 2007).

The content of total free amino acids in the sow reproductive tract fluid is shown in **Table 5**. As shown, both oviductal and uterine fluids contain lower amounts of amino acids in oestrus than in dioestrus (*Iritani et al., 1974*). The same differences between oestrus and dioestrus are known for the uterine fluid in cattle (Fahning *et al*., 1967). The investigations of Booth *et al*. (2005b) have also shown that the net rates of depletion and uptake of amino acids by pig embryos vary among amino acids and the day of embryo development. These cyclic changes in the concentration of amino acids imply that their demand differ between both gametes before fertilisation and in the early embryo (Iritani *et al.*,1974).

Table 5. Content (mM) of total free amino acids in sow reproductive tract (modified from *Iritani et al., 1974*)

Amino acids	Oviduct fluid		Uterine fluid	
	Oestrus	Dioestrus	Oestrus	Dioestrus
Alanine	0.26 ± 0.02	0.31 ± 0.11	0.28 ± 0.04	0.94 ± 0.31
Arginine	0.08 ± 0.01	0.09 ± 0.02	0.06 ± 0.03	0.07 ± 0.01
Aspartic acid	Nd	0.02 ± 0.00	0.03 ± 0.01	0.04 ± 0.01
Cystine	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.01
Glutamic acid	Nd	0.14 ± 0.02	0.21 ± 0.02	0.39 ± 0.10
Glycine	0.38 ± 0.10	0.57 ± 0.11	0.40 ± 0.11	1.32 ± 0.30
Histidine	0.06 ± 0.00	0.06 ± 0.02	0.07 ± 0.02	0.29 ± 0.05
Isoleucine	0.07 ± 0.01	0.12 ± 0.03	0.12 ± 0.02	0.36 ± 0.08
Leucine	0.13 ± 0.01	0.11 ± 0.06	0.25 ± 0.01	0.68 ± 0.19
Lysine	0.21 ± 0.01	0.25 ± 0.08	0.23 ± 0.03	1.00 ± 0.02
Methionine	Nd	0.02 ± 0.00	0.02 ± 0.00	0.09 ± 0.03
Phenylalanine	0.07 ± 0.01	0.07 ± 0.00	0.09 ± 0.00	0.29 ± 0.04
Proline	T	T	0.01 ± 0.00	0.78 ± 0.21
Serine	Nd	0.05 ± 0.01	0.08 ± 0.00	0.06 ± 0.00
Threonine	Nd	0.08 ± 0.03	0.06 ± 0.01	0.06 ± 0.01
Tyrosine	0.05 ± 0.01	0.06 ± 0.02	0.08 ± 0.00	0.26 ± 0.06
Valine	0.14 ± 0.02	0.31 ± 0.11	0.37 ± 0.03	0.79 ± 0.05
TOTAL	1.46 ± 0.25	2.27 ± 0.63	2.37 ± 0.50	7.46 ± 2.05

Nd: not detected; T: trace amounts, less than 0.01 mM

Although the concentrations of amino acids in culture media are not the same as those found in oviductal and uterine secretions (Thompson, 2000), literature reveals that the addition of amino acids to the IVC medium enhances the development of mouse (Gardner and Lane, 1993), sheep (Gardner *et al*., 1994), cow (Steeves and Gardner, 1999; Li *et al*., 2006a) and pig (Steeves and Gardner, 1999; Yoshioka *et al*., 2002; Bebee *et al*., 2007, 2009) embryos to the blastocyst stage, and increases their subsequent viability. However, the concentration, types and time when the amino acids are included in the culture medium are essential to allow them performing their functions properly. Amino acids have been grouped by Eagle (1959) into essential (EAA) and non-essential (NEAA), and are normally added to culture media as commercial concentrated stock solutions prepared at a dilution of 1:50 for EAA and 1:100 for NEAA (Beebe *et al*., 2009). Early cleavage development was found to be stimulated by the addition of NEAA to the culture medium but inhibited by EAA in both mouse (Lane and Gardner, 1997) and cow embryos (Steeves and Gardner, 1999). It is worth noting that although the presence of EAA during the first 48 h of culture in a protein-free medium inhibits the development of porcine embryos, their addition after 48 h does stimulate it (Van Thuan *et al*., 2002). Likewise, adding NEAA to the culture for the first 48 h, and NEAA and EAA (all at a 1:100 dilution) from 48 h onwards improves the cell number of porcine embryos (Beebe *et al*., 2007). Moreover, the stimulatory effect of low

concentrations of EAA has also been demonstrated in the mouse (Lane *et al*., 2001) and cow (Steeves and Gardner, 1999), but only when the EAA were added 48 or 72 h pi in mouse and cow, respectively.

The putative effects of amino acids on embryo development seem to be mediated by their important roles in culture media, including chelators, osmolytes, pH buffers, antioxidants, regulators of energy metabolism, biosynthetic precursors and energy substrates (Bavister, 1995). Moreover, the absence of amino acids and/or their addition to the culture media significantly alters the blastocyst gene expression (Ho *et al*., 1995).

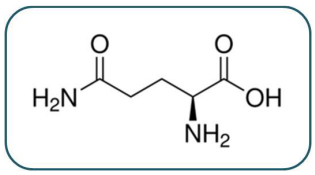


Figure 8. Chemical structure of L-Glutamine

Modified from Sigma-Aldrich Co. LLC. Copyright © 2014. Available on-line via: <http://www.sigmaaldrich.com/spain.html>

For instance, glutamine (**Figure 8**) is known to contribute to the production of ATP (Rieger *et al*., 1992) and, in pigs, it can replace glucose as the sole energy source throughout the preimplantation embryo development (Petters *et al*., 1990). Moreover, certain amino acids such as glycine and glutamine, and related compounds, may protect the embryo against variation of osmotic pressure (Biggers *et al*., 1993). This regulation of the osmotic pressure seems to be related to an increase in protein synthesis (Anbari and Schultz, 1993). Glutamine also has the property to protect the embryo from oxidative damage, helping to maintain the intracellular levels of cysteine (Bannai and Ishii, 1988).

In addition, some non-protein amino acids, such as taurine or hipotaurine (**Figure 9**), are present naturally at high concentrations in both oviductal and uterine secretions, and in mammalian oocytes and embryos (Miller and Schutz, 1987; Gardner, 1999), but they are not contained in the Eagle formulation (Eagle, 1959). For this reason, both are often used to supplement IVC media in pig, such as NCSU-23 and PZM (**Table 1**). Hypotaurine, also known as 2-aminoethanesulfonic acid, is a sulfinic acid that results from degradation of cysteine, and it is a precursor of taurine, an organic acid widely distributed through animal tissues. Interestingly, both hypotaurine and taurine also have many fundamental biological roles such as antioxidation, osmoregualtion, membrane stabilisation and modulation of calcium signalling (Huxtable, 1992). Furthermore, the

addition of taurine and/or hypotaurine to the basic NCSU-23 medium has been demonstrated to significantly improve the development of IVP porcine embryos (Petters *et al.*, 1990). Although it is known that taurine is a potent osmoprotectant, the mechanism by which this organic acid improves embryo development remains to be fully elucidated.

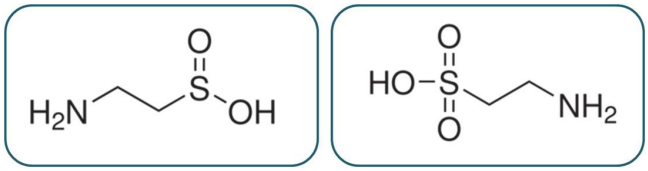


Figure 9. Chemical structures of hipotaurine (left) and taurine (right)

Modified from Sigma-Aldrich Co. LLC. Copyright © 2014. Available on-line via: <http://www.sigmaaldrich.com/spain.html>

2.2.3.1.4 Water and Inorganic Compounds

Water is the basis of all culture media since they are composed of 99% water (Loutradis *et al.*, 2000). Thus, its purity is strongly crucial as it has been related to embryonic development (Marquant-Leguienne and Humblot, 1998). Indeed, the best type of water in terms of culture media basis is double-distilled Milli®-Q water, which has the lowest concentration of inorganic ions, organic compounds as well as any pathogen (Fukuda *et al.*, 1987).

The basic components of embryo culture media, together with energy and protein sources, are salts, which dissociate into their inorganic ions in aqueous solution and form ionic nets. All embryo culture media contain the same six inorganic ions: Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, and SO_4^{2-}, and most of them also contain PO_4^{2-} (Baltz, 2012). All these inorganic compounds are carefully balanced to formulate proper culture media to more closely mimic the concentrations reported *in vivo* (**Table 2**).

2.2.3.1.5 Antioxidants

In vitro culture of embryos *per se* and/or its specific conditions may cause oxidative stress, which is, in turn, involved in many different types of cell injuries, including membrane lipids peroxidation, oxidation of amino acids and nucleic acids, apoptosis and necrosis. All these injuries may subsequently decrease the viability and efficiency of IVP of embryos (Johnson and Nasr-Esfahani, 1994; Kitagawaa *et al.*, 2004). In addition, pig embryos have a relatively high content of lipids (**Table 4**) and, thus, they are more

susceptible to peroxidation of fatty acids than other species, fact that is known to inhibit the function of cells and induce cell death (Spiteller, 2001). Therefore, protecting embryos against oxidative stress during IVC has been proposed as one of the key steps to improve mammalian embryo development and quality. Previous studies have reported that supplementing culture medium with antioxidants, such as vitamin E (Kitagawa *et al.*, 2004; Gajda *et al.*, 2008; Hossein *et al.*, 2007), L-ascorbic acid (Hossein *et al.*, 2007, Hu *et al.*, 2012; Castillo-Martín *et al.*, 2013b), or β-mercaptoethanol (Kitagawa *et al.*, 2004; Hosseini *et al.*, 2009) may be beneficial for the development of mammalian embryos, including pig, because they lead to a decrease in reactive oxygen species (ROS) levels and DNA fragmentation.

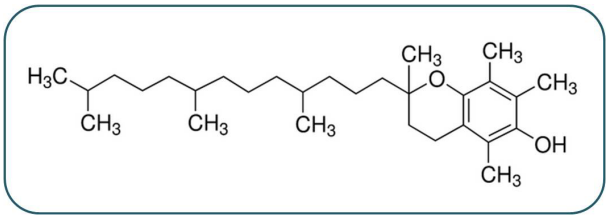


Figure 10. Chemical structure of Vitamine E or [±]-a-tocopherol

Modified from Sigma-Aldrich Co. LLC. Copyright © 2014. Available on-line via: <http://www.sigmaaldrich.com/spain.html>

Vitamin E or [±]-a-tocopherol **(Figure 10)** is the predominant lipid-soluble antioxidant in animal cells, and it is considered as a major ROS scavenger that can block lipid peroxidation in cell membranes (Tappel, 1980). For this reason, its addition to culture media has been suggested to improve the development of IVP porcine embryos. Research conducted forty years ago showed that vitamin E supplementation has a beneficial on the development of rat embryos (Steele *et al.*, 1974). Furthermore, recent studies have indicated that vitamin E supplementation increases the proportion of both parthenogenetic (Yuh *et al.*, 2010) and IVP porcine embryos reaching the blastocyst stage (Kitagawa *et al.*, 2004; Gajda *et al.*, 2008), protects chromatin integrity (Kitagawa *et al.*, 2004; Gajda *et al.*, 2008; Yuh *et al.*, 2010) and maintains triglyceride content (Romek *et al.*, 2011). Additionally, the positive effects of vitamin E on the proportion, quality and accumulation of lipids in IVP embryos have also been reported for bovine embryos (Olson *et al.*, 2000; Reis *et al.*, 2003).

L-ascorbic acid (vitamin C; **Figure 11**), the most important antioxidant in extracellular fluids, plays a key role in many biological processes such as the biosynthesis of collagen and other components of the extracellular matrix, or the protection of lipid structures against peroxidation (Rose and Bode, 1993; Buettner, 1993). Moreover, it is a potent

ROS scavenger that protects cells against oxidative damage (Rose and Bode, 1993). Whereas previous reports showed that the addition of L-ascorbic acid to culture media improves blastocyst development rates and total cell number per blastocyst, and decreases the number of apoptotic cells in IVP (Hossein *et al*., 2007), SCNT and parthenogenetic-activated (PA) porcine embryos (Huang *et al*., 2011; Hu *et al*., 2012), a recent study reported no effects on blastocyst yield, total number of nuclei or percentages of apoptosis of IVP embryos (Castillo-Martín *et al*., 2013b). In addition, despite these inconsistent results regarding developmental ability, total cell number or apoptotic indexes in IVP porcine embryos, ROS formation have been reported to be significantly lower and cryotolerance higher in embryos cultured with L-ascorbic acid than in those cultured without this antioxidant (Castillo-Martín *et al*., 2013b). Thus, adding L-ascorbic acid to IVC media seems to be an efficient strategy to enhance the ability of IVP porcine blastocysts to sustain cryopreservation and to improve their quality. Furthermore, lower ROS levels obtained in fresh blastocysts cultured with L-ascorbic acid, together with the reduction of ROS formation in vitrified/warmed blastocysts suggest that including this antioxidant is beneficial in all critical procedures, such as IVC or cryopreservation. In agreement with this, some studies have demonstrated that L-ascorbic acid is able to protect mice and cattle embryos from the detrimental effects of cryopreservation and biopsy (Lane *et al*., 2002; Korhonen *et al*., 2012).

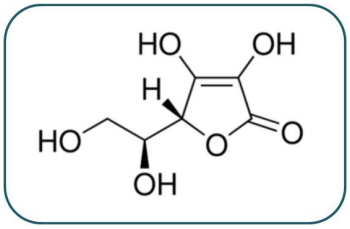


Figure 11. Chemical structure of L-ascorbic acid

Modified from Sigma-Aldrich Co. LLC. Copyright © 2014. Available on-line via: <http://www.sigmaaldrich.com/spain.html>

On the other hand, beta-mercaptoethanol (**Figure 12**) is a low molecular weight thiol compound used to reduce disulfide bonds and also used as a biological antioxidant by scavenging hydroxyl radicals (amongst others). It is often included in IVM and IVC media (Kikuchi *et al*., 2002; Karja *et al*., 2004; Castillo-Martín *et al*., 2013b) since a number of studies have indicated beneficial effects of β-mercaptoethanol during IVP of embryos (Feuagang *et al*., 2004; Kitagawa *et al*., 2004; Hashem *et al*., 2006; Hosseini *et al*., 2009). The mechanism of action through which β-mercaptoethanol exerts its effect

on embryos is not yet completely understood. Literature reveals that although β-mercaptoethanol can directly interact with some oxidized radicals and can chelate metallic ions, its main action is to protect the oxidation of cysteine, a precursor of glutathione (GSH), into cystine and increase its entry into the cell, which is known to trigger GSH synthesis (Takahashi *et al*., 1993, 2002).

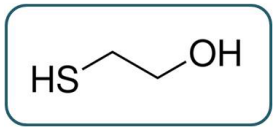


Figure 12. Chemical structure of β-mercaptoethanol

Modified from Sigma-Aldrich Co. LLC. Copyright © 2014. Available on-line via: <http://www.sigmaaldrich.com/spain.html>

It has also been reported that β-mercaptoethanol suppresses oxidative damage in the IVP embryos and improves their developmental ability. For IVP porcine embryos cultured under 20% O_2 and exposed to 50 μM β-mercaptoethanol, Kitagawa *et al*. (2004) reported higher blastocyst rates compared to those cultured without β-mercaptoethanol (57% vs. 28%). Hashem *et al*. (2006) demonstrated that supplementing KSOM and NCSU-23 media with L-ascorbic acid and/or β-mercaptoethanol improves developmental competence and quality of IVP porcine embryos. In contrast, Castillo-Martín *et al*. (2013b) have recently observed that, although supplementing NCSU-23 culture medium with β-mercaptoethanol does not render better results in the development and quality of fresh IVP porcine embryos, it does slightly improve the quality of *in vitro* vitrified/warmed blastocysts.

Therefore, it seems that data on the effects of β-mercaptoethanol supplementation are conflicting or inconsistent regarding favourable culture conditions and/or developmental stage in which β-mercaptoethanol better supports embryo development. As such, some authors (Hosseini *et al*., 2009) have reported that β-mercaptoethanol supplementation of IVC medium promotes overall developmental competence and quality of bovine IVP embryos (as measured by the proportion of blastocyst formation, hatching, cell viability, and apoptosis) either during days 1–8 and/or 9–10 of embryo culture, while others have observed such improving β-mercaptoethanol effect when added at 4–8 cell (Geshi *et al*., 1999), 8–16 cell (Caamano *et al*., 1996) and beyond the morula stages (Feuagang *et al*., 2004).

2.2.3.1.6 Growth Factors: Insulin-Like Growth Factor-I (IGF-I)

Some studies have demonstrated the presence of several growth factors and their receptors in the oviductal fluid and epithelium, respectively, during the *in vivo* development of embryos in various species including human (Pfeifer and Chegini, 1994), cattle (Schmidt *et al*., 1994), and pigs (Chang *et al*., 2000). Moreover, growth factors including insulin-like growth factor families, colony stimulating factor-1, transforming growth factor-β, granulocyte- macrophage colony- stimulating factor, epidermal growth factor, and platelet activating factor are secreted from the uterine epithelium during preimplantation embryo development (Lonergan *et al*., 1996; De Moraes *et al*., 1999; Tiemann *et al*., 2001; Pushpakumara *et al*., 2002). These growth factors and their receptors play an important role in blastocyst development, in the regulation of cellular events and in the maternal-embryonic dialogue through an autocrine and/or paracrine manner (Kim *et al*., 2005b; Weimar *et al*., 2013).

In particular, the Insulin-like growth factor (IGF)-I is a receptor-mediated autocrine/paracrine growth/survival factor for mammalian embryo development. It has been reported that IGF-I receptor encoding mRNA is expressed in IVP porcine embryos during preimplantation embryo development, and that IGF-I improves the *in vitro* development of these embryos through its specific receptors (Kim *et al*., 2005b). Likewise, the addition of IGF-I to culture medium also enhances the development of bovine embryos to blastocyst stage (Prelle *et al*., 2001; Moreira *et al*., 2002; Block *et al*.,2003) and improves embryonic survival following transfer to recipients without altering birth weight or sex ratio of offspring (Block *et al*., 2003). Moreover, it has been suggested that the addition of IGF-I to embryo culture may enhance embryo survival by improving embryo metabolism during preimplantation period, since IGF-I increases glucose uptake and translocates glucose transporter 8 in murine blastocysts (Pantaleon and Kaye, 1996; Pinto *et al*., 2002).

2.2.3.1.7 Hormones: Insulin and Leptin

It is known that insulin stimulates granulose cell mitosis and has a positive effect on ovarian cell proliferation (Spicer and Echternkamp, 1995). Moreover, it affects both oocyte maturation and embryo development *in vitro*. In particular, Lee *et al*. (2005) reported that insulin, when added during IVC period, increases the number of IVP porcine embryos reaching the blastocyst stage (34.1 ± 2.7 vs. 23.1 ± 1.4% in control). Furthermore, the rates of blastocyst formation are dramatically increased (2-fold) when this hormone is added during both IVM and IVC periods, and when it is added together with metformin, an insulin-sensitizing agent (52.4 ± 2.7 vs. 40.4 ± 2.3 %). Therefore, it has

been demonstrated that insulin can be beneficial for the production of transferable embryos in pigs, since, alone or more efficiently in combination with metformin, it improves the oocyte maturation and preimplantation embryo development of IVP porcine embryos. The effects of insulin and metformin depend on the time length of supplementation. Moreover, the beneficial effect of insulin appears to be independent from the maturation period and is directly associated with an increase of GSH content in oocyte. Metformin, in turn, significantly enhances the action of insulin on GSH content and tyrosine kinase activity (Lee *et al.*, 2005).

Recent studies have also suggested that leptin plays an important role in embryo development (Craig *et al.*, 2005). It increases oocyte maturation *in vitro*, and its inclusion in both IVM and embryo culture media further increases blastocyst development. These results suggest that leptin has a synergistic role on both oocyte maturation and preimplantation embryo development (Craig *et al.*, 2005).

2.2.3.1.8 Hyaluronic Acid

Hyaluronic acid (HA) or hyaluronan is a nonsulfated linear glycosaminoglycan (GAG) that comprises alternating units of D-glucuronic acid and N-acetyl-D-glucosamine linked by β1-3 and β1-4 glycosidic bonds (**Figure 13**; Laurent and Fraser, 1992).

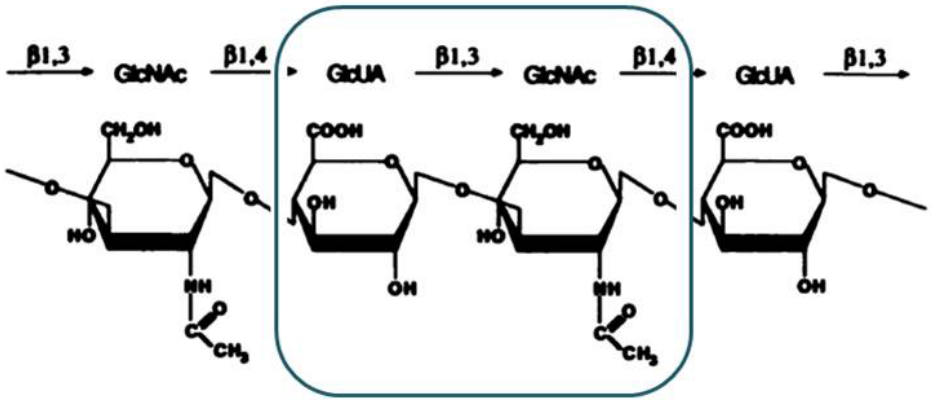


Figure 13. Chemical structure of hyaluronic acid

The polymer is built from alternating units of D-glucoronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc). Modified from *Laurent and Fraser, 1992*.

Hyaluronic acid regulates cellular events such as gene expression, signalling, proliferation, motility, adhesion, metastasis, and morphogenesis (Laurent and Fraser, 1992). As a result of the physicochemical properties of HA solutions, various

physiological functions have been assigned to them, including regulation of protein and water distribution, water-binding capacity, filtering effects and lubrication (Stojkovic *et al*., 2002). It is the major component of the extracellular matrix. It is also a normal component of mammalian folicular, oviductal, and uterine fluids (Lee and Ax, 1984; Yeste, 2003a), and it thus plays a significant role in mammalian fertilisation, implantation, extraembryonic cavitation, and embryo growth, as well as tissue morphogenesis and embryo-maternal communication (Choudhary *et al*., 2007). Moreover, it promotes differentiation of extra-embryonic tissues of murine (Hamashima, 1982) and porcine (Kim *et al*., 2005a) embryos and affects sperm motility as well as capacitation and acrosome reaction (Furnus *et al*., 2003; Yeste *et al*., 2008).

Hyaluronic acid is one of the most abundant glycosaminoglycans in the porcine female tract (0.04-1.83 $mg \cdot ml^{-1}$ and 0.32-0.59 $mg \cdot ml^{-1}$ in oviductal and uterine fluids, respectively) and their physiological concentrations increase during the peri-implantation period (Kano *et al.,* 1998). The reported reproductive physiology roles of HA on embryo development consist of increasing blastocyst development in bovine (Stojkovic *et al*., 2002; Block *et al*., 2009) and murine (Gardner *et al*., 1999) species, and the cryosurvival of bovine (Stojkovic *et al*., 2002; Block *et al*., 2009) and human (Lane and Gardner, 2007) embryos. Moreover, it enhances implantation rates and foetal development following embryo transfer in mice (Gardner *et al.,* 1999), sheep (Dattena *et al*., 2007) and humans (Lane and Gardner, 2007). In pigs, the addition of HA to the culture medium decreases the proportion of degenerated embryos and stimulates blastocyst formation of *in vivo*-fertilised embryos (Miyano *et al*., 1994) and electro-PA oocytes (Kurebayashi *et al*., 1995; Toyokawa *et al*., 2005). However, results obtained for IVP porcine embryos remain contentious. Whereas Kano *et al*. (1998) suggested that HA supports the development of porcine oocytes matured and fertilised *in vitro* to the blastocyst stage, Miyoshi *et al*. (1999) and Kim *et al*. (2005a) reported that the effects of HA on the development of IVP porcine embryos could depend on the medium and on the conditions of oocyte maturation, fertilisation and culture.

Therefore, although HA has been used to improve early embryo culture systems in pigs (Miyano *et al.,* 1994; Kano *et al.,* 1998; Miyoshi *et al.,* 1999; Kim *et al.,* 2005a), the results reported in the literature are still controversial and the mechanism by which HA improves the number and the quality of IVP embryos has not yet been clarified. Nevertheless, it seems that HA influences the development of mammalian embryos from the very early stages of embryonic development (Palasz *et al.,* 2006) through receptor-mediated pathways (Stojkovic *et al.,* 2003; Toyokawa *et al.,* 2005), by regulating the action of factor(s) synthesized by the embryo, acting in an autocrine manner (Furnus *et al.,* 2003; Toyokawa *et al.,* 2005; Palasz *et al.,* 2006) and/or regulating

the gene expression of developmentally important gene transcripts (Stojkovic *et al.*, 2003; Palasz *et al.*, 2006; Block *et al.*, 2009).

It has been described that the zona pellucida of the embryo does not prevent exogenous HA from having access to its own receptors in order to be internalized (Palasz *et al.*, 2006; Choundhary *et al.*, 2007) or to mediate a cellular response (Toyokawa *et al.*, 2005). The two main receptors known to bind HA are the cell surface receptor CD44 and HA-mediated motility/intracellular HA-binding protein (RHAMM/IHABP; Stojkovic *et al.*, 2003; Palasz *et al.*, 2006; Choundhary *et al.*, 2007).

The cell surface receptor CD44 is the principal cell membrane receptor for HA (Furnus *et al.*, 2003; Borg and Holland, 2008) and it has been identified on a wide range of cells, including all developmental stages from the germinal vesicle to the blastocyst stage in porcine parthenotes and IVP embryos (Kim *et al.*, 2005a; Toyokawa *et al.*, 2005). In contrast, CD44 expression has been detected throughout preimplantation human embryo development (Campbell *et al.*, 1995), but not in immature oocytes. CD44 is distributed throughout the cell surface and, in particular, the clustering of receptors is intensively observed in the morula and blastocyst stage parthenotes (Toyokawa *et al.*, 2005). Although a blocking approach carried out by Toyokawa *et al.* (2005) in order to interrupt the interaction between HA and CD44 did not dramatically inhibit the development of parthenotes, some of them were prevented from development by blocking HA-CD44 interactions. Thus, it is suggested that CD44 may play a role in HA action on parthenogenetic embryo development, along with the action of other factors or receptors such as RHAMM/IHABP (Stojkovic *et al.*, 2003; Toyokawa *et al.*, 2005). The expression of the cell surface RHAMM/IHABP receptors particularly disappears when cell-to-cell contact increases. However, addition of extracellular HA promotes the rapid appearance of RHAMM/IHABP at the cell surface (Palasz *et al.*, 2006).

On the other hand, it has been suggested that due to its high molecular weight and structure, HA might fill a finite space around the embryo, thereby allowing the embryo to access to maximal concentrations of self-derived growth factors. In turn, the growth factors and cytokines act in autocrine manner on embryos (Gandolfi, 1994, Palasz *et al.*, 2006). Moreover, HA may also provide a unique environment for embryos to synergistically interact with each other via paracrine growth factors in the medium, which has been demonstrated to be important for the development of the early embryo (O'Neil *et al.*, 1997).

Regarding the role of HA in the regulation of the gene expression of developmentally important gene transcripts, exogenous HA significantly increases the expression of *IGF-II* and glucose transporter I (*GLUT-1*) genes, as well as it reduces the expression of

apoptosis regulator (*BAX*), oxidative stress (*SOX*) and embryo cell-to-cell adhesion associated with embryo compactation (*ECAD*) genes in bovine (Palasz *et al*., 2006; Block *et al*., 2009). Thus, the addition of HA would enhance the embryo capacity for glucose transport, reduce apoptosis and oxidative stress and, thus, facilitate embryonic survival and quality of embryos cultured during all the period with glucose.

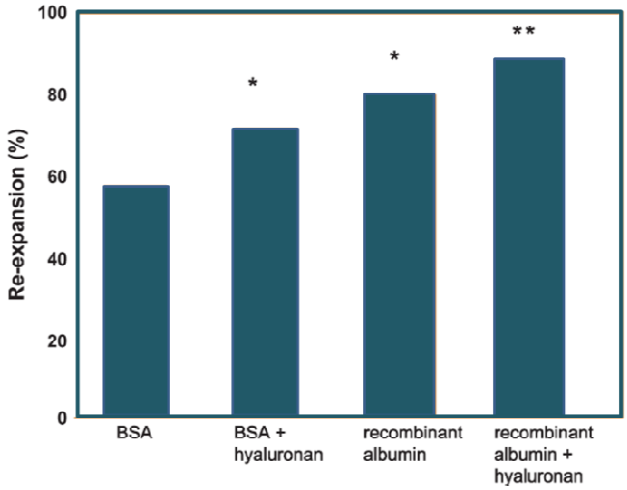


Figure 14. Effect of macromolecules in the culture medium on re-expansion after freezing and thawing of bovine blastocysts

Culture medium was supplemented with 8 mg/ml bovine serum albumin (BSA), 8 mg/ml BSA plus 0.125 mg/ml HA, recombinant albumin 2.5 mg/ml, or recombinant albumin 2.5 mg/ml plus 0.125 mg/ml HA. *Significantly different from BSA ($P < 0.05$). **Significantly different from BSA ($P < 0.01$). Modified from *Lane et al., 2003b*.

Regardless of the mechanism by which HA may improve the number and the quality of IVP embryos, HA has an important role in embryo culture media. HA can substitute for albumin in culture media but HA together with BSA of biological origin (Palasz *et al*., 1993) or recombinant BSA (Lane *et al*., 2003b) significantly increases embryo development and embryo cryosurvival (**Figure 14**). It has also been reported an improvement of blastocyst rates when both *in vivo*-derived (70% vs. 45%; Miyano *et al*., 1994) and *in vitro*-produced porcine embryos (17% vs. 0%; Kano *et al*., 1998) are cultured in IVC medium supplemented with 0.4% BSA and 0.5 mg/ml HA. However, when the concentration of BSA increases from 0.4 to 1.5%, the addition of HA does not produce this beneficial effect (Miyano *et al*., 1994). This suggests that for an unknown reason, the addition of BSA at high concentrations has a negative impact on the stimulatory action of HA. In addition, synthetic oviductal fluid (SOF) medium supplemented with HA and BSA has been reported to also enhance the development of IVP bovine blastocysts (Stojkovic *et al*., 2003). This may be related to the fact that, in

absence of serum, more RHAMM receptors on embryo cells are available to exogenous HA, than otherwise are taken by serum molecules (Palasz *et al*., 2006).

2.2.3.2 Culture Systems

Most of IVC media for porcine embryos have been used in monoculture systems. Monoculture or single systems are defined as single medium formulation that is used to support the entire embryonic development, from zygote to blastocyst stage, without reflecting any changes in the physiology of the embryo (Lane and Gardner, 2007). Nevertheless, the environment that confronts the mammalian embryo in the female reproductive tract, as well as the embryo physiology and they nutritional requirements change along the preimplantation period. Therefore, the optimal culture conditions for the development of the zygote are not the same as those for the development and differentiation of blastocysts (Gardner, 1998). Accordingly, it has been reported that the use of sequential culture systems, which are formulated to address the changing requirements of the developing embryos, improve the viability and embryonic development of murine (Lane and Gardner, 1997), human (Gardner *et al*., 1998), bovine (Lane *et al*., 2003a) and also porcine (Gandhi *et al*., 2001) embryos. These sequential culture systems seem to reduce cellular stress and limit the need for the embryo to undergo adaptation to grow up to the blastocyst stage (Lane and Gardner, 2007). As reported by Kikuchi *et al*. (2002), the sequential culture system that uses pyruvate and lactate for the first 48 h pi and, then, replaces them with glucose for the subsequent embryo development, enhances the rate of blastocyst formation and the total cell number per blastocyst compared to the monoculture system that only uses glucose as energy source. Furthermore, as described above (Section 2.2.3.1.2), a two-stage system for porcine embryo culture, in which BSA is replaced with FBS as the morula develops into blastocyst, has been suggested by some authors (Dobrinsky *et al*., 1996; Kim *et al*., 2004). Likewise, a sequential medium system containing NEAA for the first 48 h pi and NEAA and EAA for the subsequent development has been reported to stimulate development and increase cell number in porcine embryos (Van Thuan *et al*., 2002; Beebe *et al*., 2007). However, although further investigations comparing sequential and monoculture systems could better demonstrate the reliability of using the former procedures, they do not seem to be necessary (Summers and Biggers, 2003) and monoculture procedures without renewal of the medium have been reported to be a realistic alternative (Vajta *et al*., 2010).

On the other hand, it has been shown that co-culturing porcine (Kikuchi *et al*., 2002; Lloyd *et al*., 2009), bovine (Rizos *et al*., 2002) or mouse (Xu *et al*., 2004) embryos with

oviductal cells or, with medium conditioned by oviductal cells increase the success of IVP. The co-culture system was first reported for the mouse embryo in co-culture with irradiated HeLa cells (Allen and Bright, 1984). Recently, a "womb-on-a-chip" design has been developed (Mizuno *et al*., 2007). This system consists of endometrial cells grown in a lower chamber, while embryos are cultured in an upper chamber, separated from the lower by a thin membrane. In a co-culture system, somatic cells might provide unknown embryo growth promoting factor(s) and/or delete embryo toxic factor(s) from basic medium, resulting in more suitable culture conditions for embryo development (Yoshioka, 2011). However, any physical role of co-culture remains unclear, but is likely to be dependent upon co-culture cell type or species of embryos (Swain and Smith, 2011). Furthermore, a high price has to be paid for the benefits. Co-cultures have serious drawbacks, including extra work and technical difficulties with standard establishment and handling, the risk of contamination, the possible involvement of animal or human serum in phases of isolation and culture, and the lack of appropriate control over factors involved. Therefore, nowadays, with the movement towards the use of a defined media, co-culture has received less and less attention (Vajta *et al*., 2010; Swain and Smith, 2011).

2.2.3.3 Culture Conditions

2.2.3.3.1 Oxygen Tension

The oxygen environment could influence the metabolism and the oxidation-reduction potential in the embryo (Karja *et al*., 2006). The oxygen concentration in the mammalian oviduct and uterus is about 5-10% (Fisher and Bavister, 1993), whereas *in vitro* cultured embryos are usually maintained under 20% O_2 (5% CO_2 in air). However, there is a current trend towards reducing the oxygen concentration from 20% to 5% (5% CO_2 and 90% N_2), although this is still a controversial issue. Whereas some authors have suggested that low O_2 concentration (5–7%) is helpful for the *in vitro* development of embryos (Kitagawa *et al*., 2004; Karja *et al*., 2006), other reports have observed that the developmental rates and total cell numbers of blastocyst are higher in embryos cultured in an atmosphere of 20% O_2 in air than under 5% O_2 (Machaty *et al*., 1998). Likewise, other authors have also pointed out no differences under low and high oxygen concentrations in cleavage rate, blastocyst formation, total cell number and incidence of apoptosis in porcine embryos (Ock *et al*., 2005). Moreover, in *in vivo* conditions, porcine embryos reach the uterine horns, where the O_2 concentration has been reported to be lower than in the oviduct, before the compact morula stage (Fisher and Bavister, 1993). This lower oxygen tension could be partly responsible for the

switch in the production of ATP from oxidative phosphorylation to glycolysis, during compactation and blastulation (**Figure 6**). Thus, since the effect of oxygen tension on embryonic development appears to be dependent on embryo type (Booth *et al*., 2005a), the use of a sequential culture environment containing 20% O_2 up to the morula stage and 5% O_2 for later stages has been suggested (Abeydeera, 2002). However, further studies are required to better understand the exact role of oxygen and other gas tensions during *in vitro* culture on the development of porcine oocytes.

2.2.3.3.2 Communal or Individual Culture?

In the uterus, porcine embryos are surrounded by a small volume of medium (1.5 – 2 nl) since they are placed in the folds of the uterine horns. In addition, when they are in the oviduct, the amount of medium that surrounds the embryos appears to be lesser than in the uterus (probably picoliter amounts; García-Vázquez and Matás, 2010). Supporting this notion, the volume of the medium, the number of embryos cultured together and the distance between embryos are undoubtedly significant factors in the experimental design of the IVP protocols (Swain and Smith, 2011).

In various animal models, increased embryo density in smaller volumes improves development. Indeed, it has been reported that when culture of mouse (Paria and Dey, 1990; Lane and Gardner, 1992), bovine (Palma *et al*., 1992) and ovine (Gardner *et al*., 1994) embryos is performed in small volumes of medium and/or in groups, the efficiency of IVP of embryos in terms of quality and blastocyst rates, as well as survival rates after embryo transfer improve. According to Stokes *et al*. (2005), porcine embryos should be cultured in groups of 20 embryos per 10 - 20 µl of medium. In contrast, other authors have obtained better results culturing 40 – 50 embryos per 500 µl of medium (Matás *et al*., 2003). However, what appears to be clear is that the manipulation of the physical culture environment by altering embryo spacing can potentially alter the chemical environment and thus, impact embryo development (Swain and Smith, 2011).

It has been hypothesised that the beneficial effect of culturing embryos in groups and in small volume of medium is the concentration of autocrine/paracrine trophic factors secreted by the embryos in culture that may stimulate their own development and that of neighbouring embryos (Paria and Dey, 1990; Stokes *et al*., 2005Bormann *et al*., 2006; Katz-Jaffe *et al*., 2006). Accordingly, embryo culture in larger volumes would result in a dilution of the beneficial factors produced by the embryo, and their concentration in the culture medium would not be enough to perform their effects properly (Gardner, 1994). However, greater attention to detail is required with these approaches, since

conditions using low media volume may be more permissive for detrimental changes to media conditions, such as osmolality (Swain *et al*., 2010) or pH (Swain and Smith, 2011).

On the other hand, embryo development is also influenced by the distance between embryos when they are cultured together. Studies in pig and calf have shown that when embryos adhere to the bottom of culture dishes at varying distances from one to another, or using polyester mesh to control distance, embryos in closer proximity to each other improve their development (Stokes *et al*., 2005; Gopichandran and Leese, 2006; Somfai *et al*., 2010b). Indeed, the development of individual porcine zygotes to the blastocyst stage is optimal when they are cultured at a distance of between 81 and 160 µm. Therefore, as the distance between the embryos increases, blastocyst rates decline significantly, possibly because the beneficial factors produced by the embryos are diluted. Likewise, as the distance is too much reduced, such beneficial effects may be neutralized due to local high concentrations of toxic metabolites (Strokes *et al*., 2005).

2.3 Embryo Sex Determination and Selection

2.3.1 Generalities and Applications

Sex is the individual characteristic that exerts the largest impact on mammalian phenotype, growth rates and susceptibility to diseases (Ober *et al*., 2008). Moreover, as a genetic trait, sex is a singular case that cannot be manipulated by genetic selection, but only by sex predetermination methods (Seidel, 2003). Control of sex-linked hereditary diseases in human, captive management and recovery of wildlife species and, especially, genetic improvement and optimisation of livestock productivity, are some of the areas in which sex determination and selection is a very important tool.

In livestock production, the sex preselection of the offspring accelerates genetic improvement programs, increases biological and economic efficiency of productivity, and improves reproductive management due to the ability to plan matings for a specific sex (Hohenboken, 1999; Seidel, 2003; Johnson *et al*., 2005). Particularly for porcine breeding, sex determination of *in vitro* preimplantation embryos before freezing or transferring into recipient females has obvious benefits for porcine industries and commercial animal production. Indeed, in this scenario, sex determination of offspring can reduce the generation interval, accelerate genetic improvement programmes and increase the biological and economic efficiency of productivity (Johnson *et al.*, 2005). Moreover, the manipulation of the sex ratio of the offspring can improve the herd for desired purposes, in accordance with the production requirements following the market demand. Specifically, meat from boars that are nearing sexual maturity has high potential for an odour and flavour problem commonly called "boar taint". Boar taint in pork is associated with two compounds produced in the live animal: androsterone and skatole, and it refers to objectionable odour and flavour characteristics that many consumers detect in cooked pork from intact males. For this reason, castration of male livestock intended for meat production is a long-standing management practice. It helps to control the aggressive behaviour in pigs, as well as to reduce the boar taint perceived during cooking of meat mature males (Squires, 1999). However, the discussion about banning castration in male piglets is a current topic in Europe. Indeed, the European Commission welcomed the announcement that several main actors in the pig meat chain have agreed on a plan to voluntarily end the surgical castration of pigs in Europe by 1 January 2018 (European Union, 2010). Therefore, alternatives for producing higher number of females will need to be found. In

this regard, female production through sex preselection must be considered as one alternative (Johnson *et al*., 2005).

Figure 15. Woodcut illustrating the right-left theory of sex determination

This is a 16th century woodcut illustrating a statement from an Alexandrian manuscript of the 1st century A.D. stating that if a bull descends from the right side of the cow, conception will result in a bull calf, while descending from the left side will result in a heifer calf. From *Mittwoch, 1985*.

The possibility of determining the sex of the offspring before conception, both in domestic animals and in humans, is an idea that has occupied the mind of human beings for thousands of years. Debates on the origin of sexes took place long before the beginning of the scientific era and provide an insight into the culture of the times (Mittwoch, 2005). Different cultures have believed in diverse methods to control the sex of the offspring. Greek philosophers firstly suggested sidedness as the cause 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 (Bermejo-Alvarez, 2010). However, details differed. Parmemides (515 B.C.) proposed that the sex of the embryo was determined by its position on the right or left side in its mother's womb: males are on the right, females on the left; whereas Anaxagoras (500 to 428 B.C.) thought that the side of the father's testis was the deciding factor: semen from the right side becoming male and from the left side female (Mittwoch, 2005). Although the criticisms on this left-right theory, it withstood for more than 2000 years. During the course of centuries, there was ample time for the right-left theory of sex determination to become simplified, popularized and applied in diverse methods for predicting and selecting the offspring (Mittwoch, 1985). An

Alexandrian manuscript of the 1st century A.D. contains the statement that if after service the bull dismounts from the cow on the right side, this will result in a bull calf, while dismounting on the left side leads to the conception of a heifer calf (Mittwoch, 1985; **Figure 15**). In Hebraic culture, Talmud (5th century B.C.) suggested that both an orgasm by the woman before the man and placing the marriage bed in a north-south direction favoured the conception of boys. Asiatic cultures believed in an influence of the astronomy on sex ratio. 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 (Bermejo-Alvarez, 2010).

The scientific study of sex determination began in the 17th century with the discovery of spermatozoa, but the origin and function of the "spermatic animalcules" eluded investigators until 1841. The mammalian oocyte was discovered in 1827, and in the last quarter of the 19th century fertilisation was observed. Until the end of that century, the idea that sex was influenced by environmental conditions on a zygote of indeterminate sex prevailed (Mittwoch, 2005). It was believed that many environmental factors co-operate in the determination of sex, the mother's nutrition being the most decisive factor. Adverse conditions, particularly poor nutrition, but also temperature, tended to result in males, while favourable conditions result in females (Mittwoch, 2005). Environmental theories of sex determination were popular until the first quarter of the 20th century, when sex chromosomes were discovered and, thus, the theory of chromosomal determination was accepted.

According to the chromosome theory of sex determination, the genetic sex of the embryo in placental mammals is established at fertilisation with the inheritance of an X or Y chromosome from the male progenitor. Although the role of the Y chromosome in mammalian sex determination has been known since the early twentieth century, it was not until 1959 when scientists were able to identify the region of the Y chromosome that controlled this process (Hake and O'Connor, 2008). The Y chromosome, through the testis-determining gene *SRY*, acts dominantly to trigger differentiation of testes from the indifferent gonads that would otherwise develop as ovaries (Gubbay *et al*., 1990; Sinclair *et al*., 1990). The gene *Sry* is essential to establish male development, as shown by transgenic experiments where XX mice carrying the *Sry* gene develop as males (Koopman *et al*., 1991). Conversely, mutations in *Sry* or *SRY* can lead to relatively normal female development of XY mice and humans, respectively (Gubbay *et al*., 1992; Hawkins *et al*., 1992). Nevertheless, research has provided data to convince that this theory is only part of the truth. In addition to the Y chromosome, a multitude of other genes located in more than one autosomal chromosome or even in the X

chromosome, growth factors and hormones are found to be involved in sex determination (Karkanaki *et al*., 2007). The gender identity is the final result of genetic, hormonal and morphologic sex (**Figure 16**). Sex-determining process is set in motion only during the period of organogenesis when the gonads develop. Before gonad differentiation occurs, male and female preimplantation embryos display phenotypic differences such as in the epigenetic status and in the expression level of both X-linked and autosomal genes. These differences are attributed to the different sex chromosome dosage that reflects sex-related phenotypic differences under some culture conditions, including differences in speed of embryo development, survival after vitrification, cell number at the blastocyst stage and metabolism (Bermejo-Alvarez *et al*., 2010a). Conversely, once the gonads begin to differentiate as testes or ovaries, they secrete factors, notably anti-Mullerian hormone and testosterone from the testes, which determine the sexual development of the rest of the embryo (Hake and O'Connor, 2008; Bermejo-Alvarez *et al*., 2010a). Indeed, sexual dimorphism in foetal and adult tissues is mostly attributable to sex-related hormonal differences (Bermejo-Alvarez *et al*., 2010a).

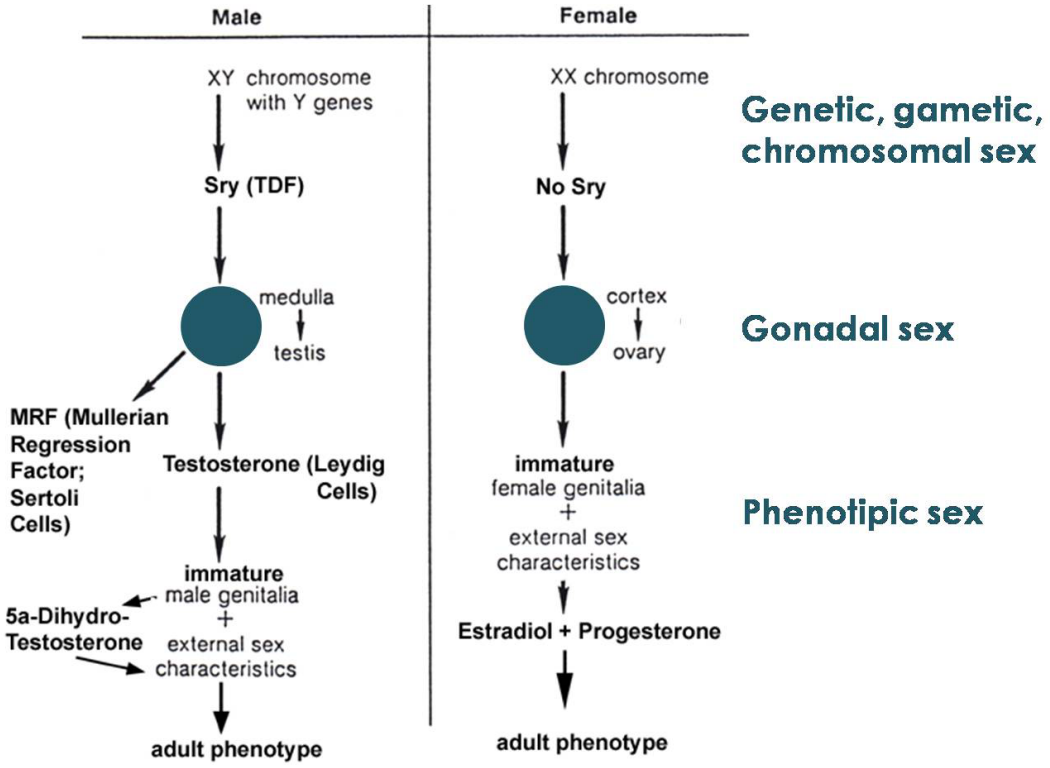


Figure 16. Process of sex determination in mammals

Modified from Memorial University of Newfoundland. Copyright © 2014. Available via: <http://www.mun.ca/biology/scarr/4241_Devo_MammalSex.html>

2.3.2 Sex Determination Methods

After the approval of the chromosome theory as the determinant of genetic sex, and the interest in the possibility of manipulating the sex ratio of the offspring of both human and domestic animals, numerous techniques for sex determination have been developed. They can be divided in two groups depending on the time of its performance: preimplantational and postimplantational techniques. At the same time, the former can be applied after or before fertilisation.

Postimplantational techniques include the identification of foetal gender using ultrasonography or metaphase karyotyping of foetal cells obtained by amniocentesis or after sampling the chorionic villi. Both techniques have certain limitations because they allow the identification of the sex but not its selection, unless using the selective abortion. Moreover, although the ultrasonography is useful and commonly used in monotocous species, it has no interest in polytocous ones, such as the pig (Fricke, 2002). Likewise, the main application of metaphase karyotyping is the diagnosis of chromosomal abnormalities in humans, and it is not currently used in domestic animals because it is a time-consuming and a technically rigorous procedure for widespread use (Larrabee *et al*., 2004).

Conversely, preimplantational techniques such as sperm sexing by flow cytometry (performed before fertilisation) and sex determination by embryo biopsy (performed after fertilisation) are currently the only reliable methods to obtain offspring of a determined sex (Niemann *et al.*, 2003; Johnson *et al.*, 2005; Naqvi, 2007). The former in the pig involves separating X- from Y-chromosome bearing sperm using flow cytometry and sperm sorting, followed by the use of this sorted sperm for AI or IVF with subsequent ET (Jonhson *et al*., 2005; Bussalleu and Torner, 2013). It has been reported that piglets have been successfully born after ET of IVF- or ICSI-derived embryos originated from sex-sorted spermatozoa (Rath *et al*., 1997; Abeydeera *et al*., 1998; Probst and Rath, 2003), or after AI (Rath *et al*., 2003; Vazquez *et al*., 2003; Grossfeld *et al*., 2005). Moreover, it has recently been reported that there is no increase in the incidence of abnormalities and in the frequency of the mutagenic indexes in the offspring produced with sorted spermatozoa (Parrilla *et al*., 2004). However, in spite of published results reporting the effectiveness of this current sexing process, in practice, sperm sexing by flow cytometry has severe limitations. The small number of cells that can be sorted per hour, the high cost of the equipment and the lower fertility that has been demonstrated when compared to non-sorted semen are some of those limitations (Johnson *et al*., 2005; Bussalleu and Torner, 2013).

In contrast, the application of embryo sex determination techniques, together with vitrification and ns-ET, may be a potential alternative for commercial purposes in swine industry. Some of the approaches that have been used to determine the sex of mammalian embryos include: (1) H-Y antigen detection, (2) X-linked enzymatic determination, (3) karyotyping, and (4) detection of Y-chromosome specific sequences by PCR or *in situ* hybridation.

The use of X-linked enzymes and a serological assay involving H-Y antigen are non-invasive methods which have the advantage of allowing all sexed embryos maintaining the integrity of the embryo, so that it is less likely that these methods impair the potential for successful embryo transfer and implantation (Bredbacka, 2001). However, these methods are not always accurate enough and they have not been applied yet in porcine embryos. Furthermore, X-linked enzymatic determination of glucose-6-phosphate dehydrogenase (G6PD; Williams, 1986), or hypoxanthine phosphoribosyl transferase (HPRT; Monk and Handyside, 1988) in mouse embryos, requires embryos to be sexed before they undergo X chromosome inactivation, hence limiting this approach to cleavage and morula stage embryos (Bredbacka, 2001). Despite a reported accuracy of over 80% in sex determination assays involving H-Y antigen detection approach in bovine embryos, this method has not been proven yet to be reproducible (Veerhuis *et al*., 1994; Gardón *et al*., 2004). On the other hand, the accuracy of karyotyping, which also allows detecting chromosome aberrations simultaneously, is nearly always 100% (Edwards and Gardner, 1967; van Vliet *et al.*, 1989). However, its utility is limited because effective karyotyping depends on harvesting cells in metaphase, which is inefficient as biopsies would at best contain only a few such cells (van Vliet *et al.*, 1989). Furthermore, the procedure requires trained cytogeneticists and is time-consuming. Another procedure is fluorescence *in situ* hybridisation (FISH), in which chromosome specific DNA probes are hybridized to nuclei only in those parts of the chromosomes with high degree of sequence complementarity. This method has been used for sexing human (Ried *et al*., 1992; Munné *et al*., 1993; Harper *et al*., 1994), bovine (Kobayashi *et al*., 1998) and porcine (Kawarasaki *et al*., 2000) embryos but, although the accuracy of this approach is acceptable, it is extremely technically challenging.

The low efficiency, accuracy and speed of all these methods preclude their routine application in the field (van Vliet *et al.*, 1989). Conversely, PCR-based assays have undergone rapid development and their use has become increasingly prevalent in commercial sexing of embryos, because of their simplicity, sensitivity, specificity, speed and cost-effectiveness (Shea, 1999; Bredbacka, 2001). Indeed, the potential of this method has been demonstrated in several species, including humans (Handyside *et al*.,

1989), cattle (Park *et al.,* 2001; Bermejo-Alvarez *et al.,* 2008a; Rattanasuk *et al.,* 2011), horses (Peippo *et al.,* 1995), mice (Kunieda *et al.,* 1992), and pigs (Pomp *et al.,* 1995; Sathasivam *et al.,* 1995), although in this latter case with a lesser extent. Molecular genetics in sexing techniques are poorly developed in swine, mainly because of the few genes and sequences that, so far, have been sequenced and mapped from the porcine genome (Niemann *et al.,* 2003).

Some of the few developed PCR-based methods for determining sex of porcine embryos involve either: (1) the co-amplification of Y chromosome single copy-specific sequences in male genomic DNA, such as the *SRY* gene, and that of specific sequences of the zinc finger genes, *ZFY/ZFX,* in both male and female embryos as an internal control to exclude misdiagnosis (Pomp *et al.,* 1995; Sathasivam *et al.,* 1995); or, (2) the amplification of single copy homologous fragments from both sex chromosomes, such as the amelogenin gene (*AMEL;* Fontanesi *et al.,* 2008; Sembon *et al.,* 2008). In spite of their reported potential, the use of multiplex or single PCR is less sensitive and specific when one needs to determine the sex from small amounts of DNA, such as the DNA contained in an embryo or a blastomere. In these cases, two-step nested PCR (duplex nested PCR) procedures have been developed to achieve high sensitivity and specificity in sex determination of human (Chong *et al.,* 1993), murine (Greenlee *et al.,* 1998) and buffalo (Appa Rao and Totey, 1999; Fu *et al.,* 2007) embryos. However, the nested primer PCR strategy requires more cycles of amplification and higher sample handling; thus, the time needed to complete this assay increases, as does the risk of contaminating samples. Another reliable and sensitive PCR-based procedure for sex determination when using small amounts of DNA is PCR with primers from specific and repetitive sequences, rather than single copy ones. Due to the presence of a large number of copies of the target sequence(s), only one step of PCR is needed, and the risk of contaminating samples decreases while sensitivity increases (Akane *et al.,* 1992). Duplex PCR methods for the amplification of both male-specific repetitive sequences and repetitive sequences from both sexes have been successfully developed to determine the sex of buffalo (Manna *et al.,* 2003) and bovine embryos (Park *et al.,* 2001; Bermejo-Alvarez *et al.,* 2008a; Carneiro *et al.,* 2011). However, this method has not yet been used for determining sex in porcine embryos.

2.3.3 Embryo Sex Dimorphism and Sex Ratio Skews

Spermatogenesis comprises the pass of primary diploid spermatogonia to haploid spermatids (meiosis) and, after further modifications, to spermatozoa (differentiation or

compartmentalisation), producing an equal amount of X and Y-bearing spermatozoa (Bonet *et al.*, 2000; Fàbrega, 2012). Assuming that X- and Y-bearing spermatozoa display equal survival/transport in the female genital tract and equal fertilizing ability, and that both sexes are subjected to an equal mortality rate, sex ratio should be 1:1. Sex ratio is the proportion of males to females in a given population at a specific stage in life between conception and death. Under normal circumstances, all mammalian species display a sex ratio roughly similar to 1:1 at birth (Bermejo-Alvarez, 2010). However, there is an extensive literature on the extent to which the embryo sex ratio can be modified by environmental factors such as nutritional environment *in vivo*, as well as by composition and conditions of embryo culture media *in vitro* (Rosenfeld and Roberts, 2004).

Environmental factors, mainly the abundance and availability of food but also the seasonal variability, have been suggested to affect maternal physiology and behaviour by impacting directly on reproductive success and offspring survival, with sex selection acting as an adaptative physiological response (Cameron, 2004; Gardner *et al*., 2010). Mice have been observed with decreased fertility and offspring sex ratios skewed toward males in summer and females in winter months, supposedly to promote male survival during territory searching in the lead up to winter when females would be more abundant (Drickamer, 1990). On the other hand, it has been observed that temporarily malnourished female mice (Rivers and Crawfor, 1974; Meikle and Drickamer, 1986) and golden hamster (Huck *et al*., 1986, 1988) deliver litters with a higher number of females. Moreover, a variety of nutritional regimens have been shown to result in a skewing of sex ratio (Gardner *et al*., 2010). While pregnant mice fed with a high fat diet tend to favour male offspring, a high carbohydrate diet shifts the ratio in favour of females (Rosenfeld *et al*., 2003). In a similar trend, food-restriction during the last week of lactation in primiparous sows causes a selective decrease in the survival of female embryos and limits the growth of all surviving embryos (Vinsky *et al*., 2006; Oliver *et al*., 2011). Moreover, in rats, a maternal diet high in sodium and potassium but low in calcium also affects the sex ratio of offspring towards females (Cluzan *et al.*, 1965; Bird and Contreras, 1986). Other maternal features such as her corporal condition, dominance status, testosterone level, stress, age, parity and litter size, and some female-linked parameters such as time of insemination, side of ovulation and exposure to contaminants have also been suggested to alter sex ratio (Bermejo-Alvarez, 2010). In this context, sex ratio skewing might represent a physiological response of the organism to changes in the intrauterine environment and maternal physiology, as a result of different nutritional regimens or other environmental conditions (Gardner *et al*., 2010).

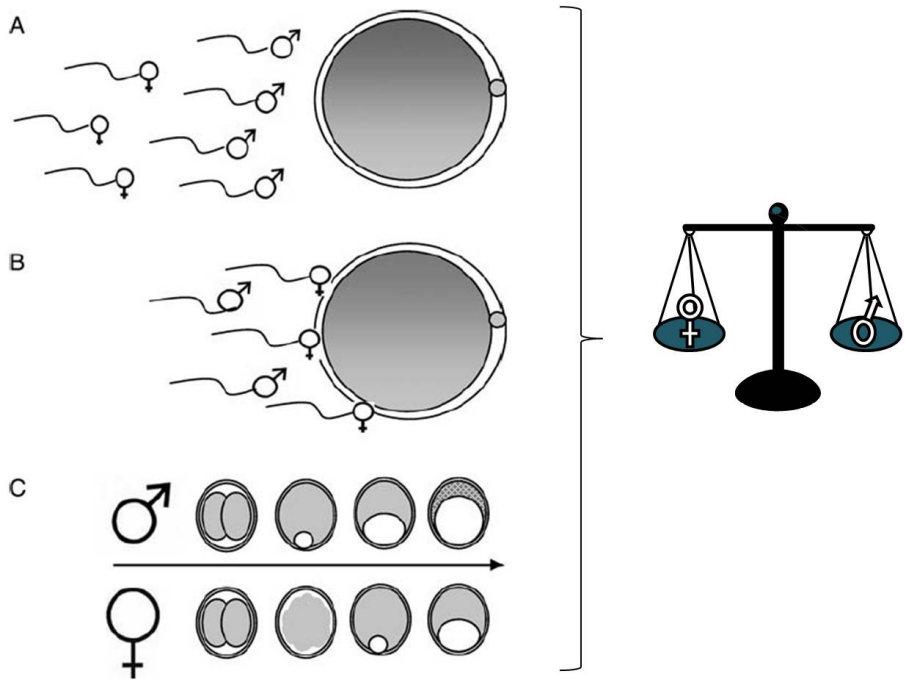


Figure 17. Biological mechanisms during fertilisation and preimplantation embryo development that could promote sex ratio skewing

(A) The ability of the reproductive tract to preclude or retard the progression of one sex-specific population of sperm could influence the outcome of fertilisation (diagrammatically in the case of X-bearing sperm, allowing the oocyte access to larger proportions of Y-bearing sperm. (B) The oocyte may preferentially select one population of sperm during sperm attachment, further influencing the outcome of fertilisation. (C) The ability of a particular population of embryo to undergo selective implantation. Modified from *Gardner et al., 2010*.

Several mechanisms, despite none of them being rigorously tested, have been hypothesized to explain skewing of sex ratios in mammals (**Figure 17**). The proposed mechanisms are not necessarily mutually exclusive and might well vary among species. They fall into two classes: those that operate prior to fertilisation (preconceptional mechanisms), or those that favour one sex over the other after fertilisation (postconceptional mechanisms; Rosenfeld and Roberts, 2004). Preconceptional mechanisms may involve either the oocyte or the spermatozoa. Regarding the former, a putative preferential selection of X- or Y-bearing spermatozoa by cumulus-oocyte complex during sperm attachment would influence the outcome of fertilisation. In this regard, it has been reported that the oocyte ability for selective processing of X- or Y-bearing spermatozoa may be maturational status-dependent, and that delaying insemination allows metaphase II (MII)-arrested bovine oocytes to be fertilised by the Y-bearing spermatozoa more efficiently (Dominko and First, 1997; Gutiérrez-Adán *et al.*, 1999; Agung *et al.*, 2006). Moreover, the time of ovulation in relation to insemination has

been reported to also promote preferential binding of X- or Y-bearing sperm in sheep embryos (Gutiérrez-Adán *et al.*, 1999). Significant differences in the sex ratio were obtained when the ewes were inseminated during the 5 h preceding ovulation (more females) with those inseminated during the 5 h after ovulation (more males). Similarly, the level of testosterone in the bovine follicular fluid has been positively correlated with the likelihood of the oocyte from being *in vitro* fertilized by Y-bearing spermatozoa (Grant *et al.*, 2008).

On the other hand, conditions prevailing in the reproductive tract of the inseminated female, e.g. state of cervical mucus, nutrient/energy status of tract secretions or vaginal pH relative to the precise time at which copulation occurred in relation to oestrus could preclude or delay the progression of one sex-specific population of sperm and, therefore, influence the outcome of fertilisation (Gardner *et al.*, 2010). Moreover, differences between X- and Y-bearing spermatozoa in motility, viability/survival, acrosome exocytosis, and/or interaction with female genital tract could result in different chances to reach the fertilisation site (Bermejo-Alvarez, 2010). Although the results are contentious, Y-bearing spermatozoa have been suggested to be more motile, or at least they display higher linearity and straightness than X-bearing in humans and cattle (Goodall and Roberts, 1976; Penfold *et al.*, 1998). Moreover, Kochhar *et al.* (2003) and Iwata *et al.* (2008) have suggested that X-bearing spermatozoa survive longer than their Y-bearing counterparts, since a reduced co-incubation time in bovine causes a skew in the sex ratio toward males.

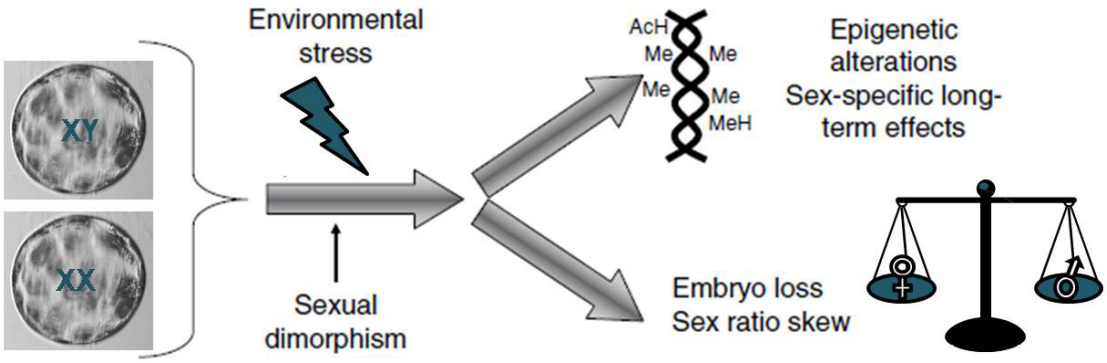


Figure 18. The effect of environmental hazards on the embryo

The effect of environmental hazards on the embryo may have no consequences, generate epigenetic alterations or affect embryo survival. Due to transcriptional sexual dimorphism, male and female genomes may react differently to environment stress, leading to sex-specific long-term effects, as epigenetic marks may be altered in a sex-specific way, or altering sex ratio, as embryo survival rates may depend on sex. Modified from *Bermejo-Alvarez et al., 2011*.

Sex ratio may also be modified by postconceptional mechanisms. These involve selective loss of embryos or foetuses of one sex with respect to the other, due to their biological differences and their different capacity to adapt to a given situation (Bermejo-Alvarez, 2010; **Figure 18**). The basis of sex-related differences (e.g., promptness of embryo development, survival and response to environmental conditions) seems to be associated with transcriptional sexual dimorphism in epigenetic, regulatory or metabolic embryo pathways. Therefore, male and female embryos are not only different in their chromosomal complement, but also in their proteome and subsequent metabolome (Bermejo-Alvarez *et al*., 2008b; Gardner *et al*., 2010).

Gene-expression pattern between male and female embryos has been compared by several research groups, and higher expression levels of X-linked genes in female embryos has been revealed when compared to male embryos (Ross *et al*., 2005; Kobayashi *et al*., 2006; Bermejo-Alvarez *et al*., 2010b). Accordingly, it has been reported that glucose-6-phosphate dehydrogenase (*G6PD*), phosphoglycerate kinase (*PGK*) and hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) exhibit higher expression and enzyme activity in female mouse embryos than in male ones, prior to X-inactivation at the blastocyst stage (Epstein *et al*., 1978; Krietsch *et al*., 1982; Williams, 1986). Similarly, in the bovine, *HGPRT*, *G6PD* and X-linked inhibitor of apoptosis protein (*XIAP*) are up-regulated in female blastocysts compared with their male counterparts (Guitérrez-Adán *et al*., 2000; Jiménez *et al*., 2003). The increased expression of these X-linked genes may explain why female embryos are less sensitive to oxidative stress (Perez-Crespo *et al*., 2005) and they have an enhanced survival under hyperglycemic conditions (Gutiérrez-Adán *et al*., 2001). In addition, the double X dosage in females and the presence of a Y-chromosome in males not only determines the transcriptional level of sex chromosome-encoded genes, but also imposes an extensive transcriptional regulation upon autosomal genes**.** Thus, they are up-regulated and co-expressed with X-linked genes in a sex related manner, thereby determining early sexual dimorphism and the beginning of sex determination and differentiation (Bermejo-Alvarez *et al*., 2010a)**.** Likewise, a variety of imprinted genes are also not expressed or methylated identically across the sexes (Rosenfeld and Roberts, 2004; Bermejo-Alvarez *et al*., 2008). A higher transcription for the epigenetic-related enzymes DNA methyltransferase 3a (*DNMT3A*) and 3b (*DNMT3B*), hnRNP methyltransferase-like 2 (*HMT1*) and interleukin enhancer binding factor 3 (*ILF3*) has been found in male embryos compared with females. Moreover, male embryos have a higher methylation level for variable number of tandem repeats (VNTR) sequence (Bermejo-Alvarez *et al*., 2008b).

As a result of transcriptional sexual dimorphism of some genes, male and female embryos have different metabolic requirements. Female bovine (Tiffin *et al*., 1991) and

mouse embryos (Lane and Gardner, 1996) have been reported to exhibit significantly higher glucose uptake than their male counterparts. This trend is consistent with the fact that female embryos also possess higher relative activity of PPP as well as higher levels of G6PD, the X-linked and limiting enzyme in the PPP (Williams, 1986; Tiffin *et al.*, 1991). Moreover, fold changes for different transcripts involved in PPP and glycolysis have been described between male and female bovine embryos (Gutiérrez-Adán *et al.*, 2000; Bermejo-Alvarez *et al.*, 2011; **Figure 19**).

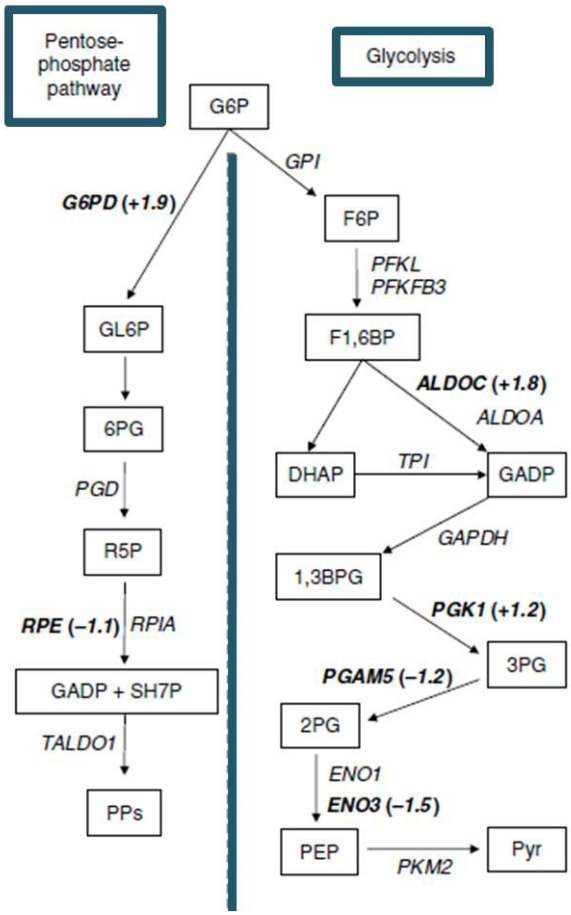


Figure 19. Fold changes between male and female embryos for different transcripts expressed in bovine blastocysts involved in pentose phosphate pathway (left) or glycolysis (right)

Different substrates are framed; the transcripts detected in blastocysts are in italics, with those exhibiting sexual differences in bold letters with their fold change: positive values are up-regulated in females and negative in males. Modified from *Bermejo-Alvarez et al., 2011*.

Mitochondria have also been proposed to play a role in sex-related metabolic differences and susceptibility to oxidative stress due to their critical role in oxidative glycolysis and in preimplantation development (Mittwoch, 2004). In this sense, it has

been observed that mtDNA copy number is higher in male bovine blastocysts than in female blastocysts (Bermejo-Alvarez *et al*., 2008b), and the cellular components "mitochondria" and "mitochondrial inner membrane" are over-represented in the up-regulated genes in male embryos (Bermejo-Alvarez *et al*., 2010b).

On the other hand, amino acid metabolism has also been shown to be different between male and female bovine embryos cultured *in vivo* and *in vitro* (Sturmey *et al*., 2010). Differences between XX and XY embryos were observed in the profiles of seven amino acids (arginine, glutamate, methionine, glycine, phenylalanine, tyrosine and valine) in blastocysts derived *in vitro*, and also of two amino acids in blastocysts derived *in vivo* (glycine and valine). When cultured *in vitro*, whereas an increased depletion of arginine, glutamate and methionine, and an increased appearance of glycine were found in female embryos, male embryos displayed an increased depletion of phenylalanine, tyrosine, and valine. Valine depletion by *in vivo* derived cattle blastocysts was similar to that seen *in vitro*, with XX embryos depleting this amino acid to the greatest extent. Moreover, glutamine has been shown to influence PPP metabolism of glucose to a greater extent in female bovine embryos, as it works as an alternative source for carbohydrates (Tiffin *et al*., 1991).

Another frequently reported difference between early male and female embryos concerns the cleavage rates in the first days after fertilisation. During *in vitro* culture, porcine (Dang-Nguyen *et al*., 2010; Somfai *et al*., 2010a), bovine (Avery *et al*., 1992; Yadav *et al*., 1993; Lonergan *et al*., 1999; Kochhar *et al*., 2003), mouse (Peippo and Bredbacka, 1995), sheep (Bernardi and Delouis, 1996) and human (Ray *et al*., 1995) male embryos tend to develop faster and earlier reach more advanced stages of development than female embryos. Male *in vivo*-produced porcine embryos, both prior to and after blastocyst hatching, are larger and have more cells than female embryos (Cassar *et al*., 1995). However, other studies, as those of NgE *et al*. (1995) in humans, Hollm *et al*. (1998) in cattle, and Pomp *et al*. (1995) and Kaminski *et al*. (1996) in pigs did not find differences between male and female embryos when reached the blastocyst stage. These conflicting findings may be due to within-cohort heterogeneities in embryo kinetics, and subsequent difficulties of either sex embryo to be cultured optimally *in vitro*, as well as to the variability in the source of donor sperm (Alomar *et al*., 2008; Gardner *et al*., 2010). Conversely, since *in vitro* production of embryos, rather than *in vivo*, provides most of the evidence of faster development of male embryos, some authors have speculated that this sex-bias may be simply an artefact of *in vitro* culture (Peippo and Bredbacka, 1995) or a result of invasive embryo manipulation (Wrenzycki *et al*., 2002).

In this regard, it has been proposed that certain *in vitro* culture conditions affect the sex ratio of IVP embryos by influencing the embryo development of a given gender (Bermejo-Alvarez *et al.,* 2011). Animal and human studies have reported that carbohydrate and amino acid metabolism are closely linked to embryo developmental competence and viability (Gardner *et al*., 2000). Related to this, it is worth noting that manipulating the metabolic profile of the embryos during *in vitro* culture has an impact on *in vitro* development, first zygotic cleavage, gene expression, cryopreservation and sex ratio in mammalian embryos (Holm *et al*., 1998; Lonergan *et al*., 2000; Rubessa *et al*., 2011; Castillo-Martín *et al*., 2013a). Intriguingly, Yadav *et al*. (1993) suggested that fluctuations in the culture environment during the first 48 h pi. may cause a preferential loss of bovine female embryos by 120 h pi., while Larson *et al*. (2001) observed that a block to female bovine embryo development in a glucose-containing medium occurred when the blastocoels' cavity began to form. In a similar trend, the cohort of females that fail to advance to expanded blastocyst appear to be less tolerant to high glucose concentrations in the medium than the successful females (Larson *et al*., 2001). On the other hand, the addition of glucosamine to the *in vitro* culture (IVC) medium significantly decreases bovine embryo development and skews the sex ratio towards males (Kimura *et al.,* 2008).

Part III
Article Compendium

Chapter 3

Paper I

Sex determination of porcine embryos using a new developed duplex polymerase chain reaction procedure based on the amplification of repetitive sequences

Eva Torner, Eva Bussalleu, Maria Dolors Briz, Alfonso Gutiérrez Adán, Sergi Bonet

Reproduction, Fertility and Development

2013:25(2):417-425

doi: 10.1071/RD12033

Torner E, Bussalleu E, Briz MD, Gutiérrez-Adán A, Bonet S. 2013. Sex determination of porcine embryos using a new developed duplex polymerase chain reaction procedure based on the amplification of repetitive sequences. *Reproduction Fertility and Development* 25(2) : 417-425

http://dx.doi.org/10.1071/RD12033

http://www.publish.csiro.au/?paper=RD12033

Submitted: 11 February 2012

Accepted: 31 March 2012

Published: 14 May 2012

Abstract

Polymerase chain reaction (PCR)-based assays have become increasingly prevalent for sexing embryos. The aim of the present study was to develop a suitable duplex PCR procedure based on the amplification of porcine repetitive sequences for sexing porcine tissues, embryos and single cells. Primers were designed targeting the X12696 Y chromosome-specific repeat sequence (SUSYa and SUSYb; sex-related primer sets), the multicopy porcine-specific mitochondrial 12S rRNA gene (SUS12S; control primer set) and the X51555 1 chromosome repeat sequence (SUS1; control primer set). The specificity of the primer sets was established and the technique was optimised by testing combinations of two specific primer sets (SUSYa/SUS12S; SUSYb/SUS12S), different primer concentrations, two sources of DNA polymerase, different melting temperatures and different numbers of amplification cycles using genomic DNA from porcine ovarian and testicular tissue. The optimised SUSYa/SUS12S- and SUSYb/SUS12S-based duplex PCR procedures were applied to porcine in vitro-produced (IVP) blastocysts, cell-stage embryos and oocytes. The SUSYb/SUS12S primer-based procedure successfully sexed porcine single cells and IVP cell-stage embryos (100% efficiency), as well as blastocysts (96.6% accuracy; 96.7% efficiency). This is the first report to demonstrate the applicability of these repetitive sequences for this purpose. In conclusion, the SUSYb/SUS12S primer-based duplex PCR procedure is highly reliable and sensitive for sexing porcine IVP embryos.

Keywords

in vitro-produced embryos; IVF; pig

Chapter 4

Paper II

Embryo development and sex ratio of *in vitro*-produced porcine embryos are affected by the energy substrate and hyaluronic acid added to the culture medium

Eva Torner, Eva Bussalleu, Maria Dolors Briz, Marc Yeste, Sergi Bonet

Reproduction, Fertility and Development

[In Press]

doi: 10.1071/RD13004

Torner E, Bussalleu E, Briz MD, Yeste M, Bonet S. 2013. Embryo development and sex ratio of in vitro-produced porcine embryos are affected by the energy substrate and hyaluronic acid added to the culture medium. *Reproduction Fertility and Development* [In press]

http://dx.doi.org/10.1071/RD13004

http://www.publish.csiro.au/?paper=RD13004

Submitted: 4 January 2013

Accepted: 23 March 2013

Published: 9 May 2013

Abstract

In the present study, the effects of replacing glucose with pyruvate–lactate and supplementing these in vitro culture (IVC) media with hyaluronic acid (HA) on porcine embryo development and sex ratio were examined. The in vitro-produced (IVP) porcine embryos were cultured in NCSU-23 medium with 0.0, 0.5 or 1.0 mg mL–1 HA, and with either 5.55 mM glucose (IVC-Glu) or pyruvate (0.17 mM)–lactate (2.73 mM) from 0 to 48 h post insemination (h.p.i.) and then with glucose from 48 to 168 h.p.i. (IVC-PL). Those embryos cultured with IVC-PL had significantly higher blastocyst rates (23.7 ± 1.5%) than those cultured with IVC-Glu (14.27 ± 2.75%). At 1.0 mg mL–1, HA tended to skew the sex ratio of blastocysts towards males in those embryos cultured in IVC-PL, and led to a significant decrease in the blastocyst rate compared with embryos cultured in the presence of 0.5 and 0.0 mg mL–1 HA and IVC-Glu (4.28 ± 0.28% vs 11.01 ± 1.42% and 10.14 ± 2.77%, respectively) and IVC-PL (14.37 ± 1.35% vs 20.96 ± 2.85% and 22.99 ± 1.39%, respectively). In contrast, there were no significant differences in the total cell number per blastocyst or in apoptosis rates. In conclusion, pyruvate and lactate were the preferred energy substrates in the early stages of IVP porcine embryos. Moreover, 1.0 mg mL–1 HA significantly decreased the percentage of blastocyst rates in both the IVC-Glu and IVC-PL groups, but only by a preferential loss of female embryos for those cultured in IVC-PL.

Keywords

Blastocyst; glucose; hyaluronan; pig; pyruvate-lactate

Chapter 5

Paper III

Energy substrate influences the effect of the timing of the first embryonic cleavage on the development of *in vitro*-produced porcine embryos in a sex-related manner.

Eva Torner, Eva Bussalleu, Maria Dolors Briz, Marc Yeste, Sergi Bonet

Molecular Reproduction and Development

2013:80:924-935

doi: 10.002/mrd.22229

Torner E, Bussalleu E, Briz MD, Yeste M, Bonet S. 2013. Energy substrate influences the effect of the timing of the first embryonic cleavage on the development of in vitro-produced porcine embryos in a sex-related manner. *Molecular Reproduction and Development* 80(11) : 924-935

http://dx.doi.org/10.1002/mrd.22229

http://onlinelibrary.wiley.com/doi/10.1002/mrd.22229/abstract

Article first published online: 10 SEP 2013

Abstract

In vitro culture conditions and certain events during the earliest stages of development are linked to embryonic survival, possibly in a sex-related manner. In vitro-produced (IVP) porcine embryos cultured with glucose (IVC-Glu) or pyruvate–lactate (IVC-PL) were tested for any relationship between the timing of the first embryonic cleavage and development and sex ratio. The embryos were assigned to IVC-Glu or IVC-PL groups and classified depending on the timing of their first cleavage: 24, 26, 30, and 48 hr post-insemination (hpi). They were cultured separately in vitro and evaluated for cleavage rate and pattern, blastocyst rate and stage, cell number, apoptosis, and sex ratio. Regardless of energy source, the percentage of two-cell stage and fragmented embryos at the time of their first cleavage was, respectively, higher and lower in early-cleaving embryos. Those embryos cleaved by 24 hpi developed to blastocysts at a higher rate (IVC-Glu: 37.90 ± 3.06%; IVC-PL: 38.73 ± 4.08%) than those cleaved between 30 and 48 hpi (IVC-Glu: 5.87 ± 3.02%; IVC-PL: 8.41 ± 3.50%). Furthermore, a shift toward males was seen among embryos first cleaved before 30 hpi, versus towards females among those cleaved later. The early-cleaving embryos, only from the IVC-PL group, had a higher proportion of expanded blastocysts (81.05 ± 6.54% vs. 13.33 ± 13.33%) with higher cell numbers than their late-cleaving counterparts. Moreover, a shift toward males only appeared at the blastocyst stage in IVC-PL embryos. These findings confirm that the timing of the first cleavage influences development of IVP porcine embryos in a sex-related manner, and it depends on the main energy source of the in vitro culture medium.

Part IV
General Discussion & Conclusions

Chapter 6

General Discussion

The ability to produce and support the development of embryos *in vitro* is required by biotechnological researchers since most of the new reproductive technologies rely on this basic procedure. Developing efficient IVP techniques in pigs will enable researchers to generate viable embryos with less cost and less time than those produced *in vivo*. In addition, it will contribute to research in reproductive physiology, agriculture and biotechnology. For instance, some applications of IVP technology have included efforts to: (1) upgrade the productive and genetic performance of animals, (2) overcome infertility of valuable high yielding animals, (3) produce transgenic and cloned animals, (4) provide a source of sexed embryos, and (5) elucidate events related to maturation, fertilisation of occytes and development of embryos at the molecular level, as these events are difficult to study under natural conditions in living animals (Kikuchi *et al*., 2002; Kątska-Książkiewicz, 2006).

The developmental competence of IVP embryos has been confirmed and improved in pigs (Yoshida *et al*., 1993; Kikuchi *et al*., 2002; Yoshioka *et al*., 2012; Castillo-Martín *et al*., 2013a) since it was first reported by Mattiolli *et al*. (1989). However, in spite of the application of various modifications to improve the quality of resultant embryos, the efficiency of the procedure and the development competence and quality of IVP embryos in pigs is rather low compared with *in vivo* counterparts, and with the *in vitro* development of other species such as bovine or mouse (Kątska-Książkiewicz, 2006; Dang-Nguyen, 2011). Approximately, only 20-30% of porcine IVP embryos develop up to the blastocyst stage (Nagai *et al*., 2006; Isom *et al*., 2012). In addition, the quality of the resultant blastocysts and pregnancy rates after embryo transfer are still low in relation to other species (Kikuchi *et al*., 1999; Jeon *et al*., 2011).

In vitro embryo production systems generally consist of three stage-specific culture environments specially designated to supply in the needs of each stage of oocyte, zygote and embryo development: (1) *in vitro* maturation (IVM), (2) *in vitro* fertilisation (IVF), and (3) *in* vitro culture (IVC). During this procedure, oocytes and embryos need to

gather many signal transduction mechanisms, cytoskeletal constituents and genes in order to successfully complete all the necessary developmental processes to produce embryos capable of generating functional cell lines or live healthy offspring (Kidson, 2004). The major reduction in *in vitro* development rates occurs during the last part of the IVP of embryos, during the IVC and between the two-cell and blastocyst stages (Rizos *et al*., 2008b). Several major developmental events occur during this period, which explain its importance in determining blastocyst quality. These events include: (1) the first cleavage division, the timing of which is known to be critical in determining the subsequent development of the embryo (Lonergan *et al*., 1999; Booth *et al*., 2007; Dang-Nguyen *et al*., 2010), (2) the activation of the embryonic genome at the four- to eight-cell stage (Jarrel *et al*., 1991; Schoenbeck *et al*., 1992), (3) compaction of the morula, which involves the establishment of the first intimate cell-to-cell contacts in the embryo (van Soom *et al*., 1997), and (4) blastocyst formation involving the differentiation of two cell types, the trophectoderm and the inner cell mass (Rizos *et al*., 2008b).

Even though the majority of preimplantation embryonic deaths remain unexplained (Isom *et al*., 2012), suboptimal *in vitro* culture conditions and inherent oocyte and zygote quality seem to be important factors that determine whether an embryo will yield to premature embryonic failure or develop appropriately (Lonergan *et al*., 1999; Somfai *et al*., 2010a). Chronic and cumulative stresses caused by suboptimal culture conditions, or insufficiencies in the constituents of the culture medium, can lead to oocyte or embryo anomalies at a genetic or metabolic level and severely jeopardise the viability of the resultant embryo (Kidson, 2004). Indeed, it has been demonstrated that the energy substrate used during *in vitro* culture affects both the production and viability of blastocysts (Rubessa *et al*., 2011). Furthermore, manipulating the metabolic profile of the embryos during *in vitro* culture may impact development, first zygotic cleavage, gene expression, cryopreservation, and sex ratio of mammalian embryos (Lonergan *et al*., 2000; Rubessa *et al*., 2011). There is emerging evidence that 10-15% of IVP mammalian embryos permanently arrest in mitosis at four-cell stage (Jeon *et al*., 2011), which is the time of embryo transport into the uterus *in vivo* (Oxenreider and Day, 1965), and it is correlated temporally with the activation of the embryonic genome (Jarrel *et al*., 1991; Schoenbeck *et al*., 1992). The *in vitro* block may be induced by insufficient culture conditions at early stages of development that prevent gene activation or suppress transcription or translation (Peters *et al*., 2001). Likewise, there is increasing awareness that timing of several events during the earliest stages of development, such as that of the first embryonic cleavage, and oocyte quality may be linked to subsequent embryonic competence (Rizos *et al*., 2002; Booth *et al*., 2007; Isom *et al*., 2012).

Many studies focused on IVC of pig embryos have tested several modifications of culture conditions. These modifications have so far included (1) different media (Wang *et al*., 1997; Machaty *et al*., 1998; Gajda and Smorag, 2004; Im *et al*., 2004), (2) different volumes of culture medium and different media preparation, i.e. medium covered with mineral oil or without covering (Yoshioka *et al*., 2002, 2003; Gil *et al*., 2003; Im *et al*., 2004, 2005), (3) different distances between embryos in embryo culture groups (Stokes *et al*., 2005), and (4) different ingredients for media supplementation (Ka *et al*., 1997; Swain *et al*., 2002; Craig *et al*., 2005; Lee *et al*., 2005; Karja *et al*., 2006; Beebe *et al*., 2009, 2013; Yoshioka, 2011). In the current thesis, two different approaches have been used to improve IVP of pig embryos, giving particular emphasis on the earliest stages of development. The focus on these specific stages is explained because key processes and pathways that are triggered in these definite moments are known to be critical and have real consequences for future embryonic development (Rizos *et al*., 2008b). In particular, in **PAPER II**, different substances were added to the culture medium; different energy sources during de first 48 h pi and different concentrations of HA during all the culture period. In **PAPER III**, intrinsic characteristics of the zygote at early stages of development, specifically, the effects of the timing of the first embryonic cleavage on subsequent embryonic competence in two different culture conditions were determined. Moreover, both approaches were also carried out to determine any sex ratio deviation of embryos cultured under these conditions using the duplex PCR technique developed in **PAPER I**. The outcomes regarding sex ratios and thus sex-related embryo survival will be discussed separately below.

The effects on the development of IVP porcine embryos of: (1) replacing glucose with pyruvate and lactate in the culture medium for the first 48 h of culture, (2) adding different concentrations of HA to the culture medium; and (3) their putative synergic effects are particularly addressed in **PAPER II**. Meanwhile, **PAPER III** was conducted to study the influence of the timing of first cleavage in the subsequent development of IVP porcine embryos cultured *in vitro* in glucose-based and pyruvate- and lactate-based NCSU-23 culture media. In fact, this is not the first time that the effects of adding HA to the IVC medium and replacing glucose with pyruvate and lactate on porcine embryo development have been studied (Miyano *et al*., 1994; Kano *et al*., 1998; Kure-bayashi *et al*., 1995; Toyokawa *et al*., 2005; Kim *et al*., 2005a). Likewise, previous studies have already proven that timing of the first cleavage has a great influence on the developmental competence of IVP porcine embryos (Booth *et al*., 2007; Dang-Nguyen *et al*., 2010). However, the original contribution of the study assessed in **PAPER II** is that conclusive data about the addition of these substances to the most successful and often used medium in the field, the NCSU-23 medium, were obtained, and the concentrations that more closely approximate the physiological concentrations of HA

and energy sources in the pig oviductal and uterine fluids were used. Moreover, it is the first time that the putative interaction between HA and energy substrates is described. To the best of our knowledge, **PAPER III** also documents, for the first time, the influence of the energy substrate on several developmental parameters among embryos that first cleaved at different time points; this influence is not always clear when embryos are evaluated, regardless of the time of their first cleavage.

Hyaluronic acid is secreted by granulose and expanding cumulus cells (Eppig, 1979; Nagyova *et al*., 2000). Moreover, it is the major glycosaminoglycan present in follicular, oviductal and uterine fluids (Lee and Ax, 1984; Suchanek *et al*., 1994; Yeste, 2013a). In pigs, the physiological concentrations of HA present in follicular, oviductal and uterine fluids range from 0.04 to 1.83 mg·ml^{-1} (16–39% of all glycosaminoglycans; Kano *et al*., 1998). As it is well understood, HA plays an important role in reproductive physiology, influencing the development of mammalian embryos from the very early stages of embryonic development (Palasz *et al*., 2006). This effect is mediated through receptor-mediated pathways (Stojkovic *et al*., 2003; Toyokawa *et al*., 2005; Kim *et al*., 2005a), by regulating the action of factor(s) synthesized by the embryo, acting in an autocrine manner (Furnus *et al*., 2003; Toyokawa *et al*., 2005; Palasz *et al*., 2006), or regulating the gene expression of developmentally important gene transcripts (Stojkovic *et al*., 2003; Palasz *et al*., 2006; Block *et al*., 2009).

In the trial described in **PAPER II**, the same maturation and fertilisation conditions were used in all treatments, but two different culture conditions for the first 48 h pi of culture (pyruvate (0.17 mM)-lactate (2.73 mM) or glucose (5.55 mM)) were also tested together with three different HA concentrations (0.0, 0.5 and 1.0 mg·mL^{-1}) in order to evaluate their effects on porcine embryo development. Previous reports in porcine IVP embryos showed that 0.5 mg·mL^{-1} HA increase the rate of blastocyst formation and the total cell number due to an increase in trophectoderm cells (Kano *et al*., 1998; Kure-bayashi *et al*., 1995; Miyoshi *et al*., 1999; Kim *et al*., 2005a). In contrast, results in **PAPER II** showed that 0.5 mg·mL^{-1} HA does not favour embryo development in terms of blastocyst rates and number of cells, but it has detrimental effects when it is added at higher concentrations (1.0 mg·mL^{-1}). It is known that the efficiency of the physiological functions of HA solutions are controlled by HA molecular weight and concentration (Palasz *et al*., 2006). Some of their physiological functions are the ability to regulate protein and water distribution (Stojkovic *et al*., 2002), and its capacity to expand its random coil causing an entanglement-structure surrounding embryos in the medium (Toyokawa *et al*., 2005). Therefore, the significantly lower blastocyst rates obtained in

the 1.0 mg·mL^{-1} HA-treated group could be explained as this concentration is too high for HA to perform its functions properly. Hyaluronic acid is a natural high viscosity mucopolysaccharide able to increase the viscosity of culture medium because of its chemical and physical properties (Stojkovic *et al.*, 2002). Thus, the viscosity of the culture medium with 1.0 mg·mL^{-1} HA could have impaired embryo access to maximal concentrations of self-derived growth factors or other components present in the medium.

On the other hand, one possible reason for the non-beneficial, but also non-detrimental effects of 0.5 mg·mL^{-1} HA on blastocyst formation and quality in **PAPER II,** might be the variable number of HA receptors at different stages of embryo development. It has been described that the number of HA receptors is low at 48 h pi, but it increases at the early morula stage (Stojkovic *et al.*, 2003; Palasz *et al.*, 2006). In the experiment performed in **PAPER II**, each concentration of HA was added to the culture medium throughout the culture period (0 h pi – 168 h pi). Thus, the beneficial effects of HA at advanced embryo stages could be offset by detrimental effects at earlier stages. Moreover, the effects of HA on porcine IVP embryos have been reported to depend on the media and on the conditions of maturation, fertilisation and culture. Exogenous HA has only been suggested to improve the development of porcine IVP embryos when such conditions are suboptimal (Miyoshi *et al.*, 1999; Kim *et al.*, 2005a). Nevertheless, the results exposed in **PAPER II** demonstrate that the primary energy substrate in the culture medium during the first 48 h pi does not influence the effects of HA on porcine embryo development, in terms of cleavage rates, blastocyst formation or total cell number per blastocyst, despite culture conditions being suboptimal, like those with glucose.

Although putative cytotoxic effects have been reported when glucose is present during early embryonic stages in pigs, most porcine embryo culture media include glucose as the main energy substrate. Culturing IVP porcine embryos in the presence of high concentrations of glucose has been demonstrated to cause oxidative stress, as glucose is metabolized through the PPP (Swain *et al.*, 2002). In addition to that, a mitochondrial abnormality caused by a downregulation of glucose transporters, enzyme inactivation, and DNA fragmentation appears to negatively impact on genome activation and/or difficult overcoming the 4-cell block (Medvedev *et al.*, 2004). Furthermore, porcine embryos have a limited ability to utilize glucose at early stages of development (Kikuchi *et al.*, 2002). The first marked increase in glucose metabolism occurs at or around the four- or eight-cell stage (Karja *et al.*, 2006), when the activation of the pig embryonic genome usually takes place (Jarrell *et al.*, 1991; Schoenbeck *et al.*, 1992). In contrast,

pyruvate and lactate are among the essential factors that may help early-stage IVP porcine embryos overcome this block (Dang-Nguyen *et al.*, 2010). They provide the embryos with more suitable conditions for balancing cellular reduction/oxidation (redox) and for preventing the detrimental effect on embryo development (Swain *et al.*, 2002; Karja *et al.*, 2006).

Taking into account that the composition of the culture medium used for the first 48 h pi has a critical effect on *in vitro* embryo potential, previous studies used alternative energy sources, such as pyruvate and lactate, during IVC. However, results concerning the beneficial effects of replacing glucose with pyruvate and lactate have been inconsistent. Several studies have shown improvements in blastocyst development and in the quality of porcine IVP embryos in terms of total cell number following the replacement of glucose with pyruvate and lactate as the energy substrate in IVC medium (Kikuchi *et al.*, 2002; Yoshioka *et* al., 2002; Karja *et al.*, 2006; Beebe *et al.*, 2007), whereas some others have reported no differences in blastocyst formation rate or cell number (Karja *et al.*, 2004; Kim *et al.*, 2004; Castillo-Martín *et al.*, 2013a). Results from **PAPER II** and **PAPER III** have demonstrated that pyruvate and lactate at the specific concentrations of 0.17 and 2.73 mM, respectively, are the preferred energy substrates at the early stages of porcine embryo development. Culture with 5.55 mM glucose during the first 48 h pi decrease both the kinetics of IVP embryo development (**PAPER III**) and the blastocyst rate (**PAPER II** and **PAPER III**) compared to culture with 0.17 and 2.73 mM pyruvate and lactate, respectively.

Lactate is known to regulate intracellular pH, the proper NAD^+:NADH ratio, and the redox equilibrium in the embryo (Iwasaki *et al.*, 1999; Swain *et al.*, 2002). It also enhances the embryotrophic role of pyruvate, an antioxidant compound that acts not only as an energy source, but can also detoxify ammonia in the embryo through transamination to alanine, which can be exported from the cell (Lane and Gardner, 2000; Swain *et al.*, 2002; Ménézo *et al.*, 2013). Findings in **PAPER II** are in agreement with other studies that used the same glucose, pyruvate and lactate concentrations, but with different basic culture media, and differ from those that used the same culture medium but with higher concentrations of pyruvate and lactate. Therefore, results of **PAPER II** suggest that specific concentrations and an optimal ratio of pyruvate and lactate are critical for regulating the oxidation-reduction equilibrium between the cytoplasm and mitochondria and for avoiding their detrimental effects on embryo development. Indeed, pyruvate and lactate at the specific concentrations tested in **PAPER II** and **PAPER III** provide better conditions at the early stages of porcine embryos to reach higher blastocyst rates at more advanced stages, but do not improve embryo quality (i.e. total cell number per blastocyst and percentage of cells with fragmented

DNA) when compared to the medium containing glucose. The intensity of the oxidative stress caused by IVC conditions and its duration can induce differential responses in cell proliferation to cell arrest or cell death by apoptosis in embryos (Karja *et al.*, 2006). Therefore, the reduced blastocyst rate and the lack of significant differences in the percentage of cell with fragmented DNA, as a mark of apoptosis, and total cell number per blastocyst when comparing embryos cultured in the presence of glucose or pyruvate and lactate could be explained by the contribution of the glucose-only culture to cell arrest rather than cell death. Another reason by which embryo cell number was not significantly different between embryos cultured with pyruvate and lactate and with glucose could be since experiments in **PAPER II** evaluated that parameter regardless of the time of the embryos' first cleavage. As **PAPER III** documents, the energy substrate has a significant effect on several developmental parameters, such as on the total number of cells per blastocyst of embryos that first cleaved at different time intervals.

Findings in **PAPER III** are consistent with previous results which reported that timing of the first cleavage has a great influence on the subsequent developmental competence of IVP porcine embryos (Booth *et al.*, 2007; Dang-Nguyen *et al.*, 2010). Those zygotes that cleave earlier after insemination are more likely to form blastocysts than their later-cleaving counterparts. This clear relationship has also been demonstrated in humans (Bos-Mikich *et al.*, 2001; Fenwick *et al.*, 2002) and in cattle (Lonergan *et al* 2000; Rizos *et al.*, 2008a; Somfai *et al.*, 2010a; Pers-Kamczyc *et al.*, 2012). Moreover, a higher proportion of blastocysts derived from early-cleaving oocytes hatch from the zona pellucida, thereby suggesting that such blastocysts are of superior quality than those derived from late-cleaving oocytes (Ward *et al.*, 2001). In **PAPER III**, successful development up to the blastocyst stage markedly decreases when both the time from fertilisation to first cleavage and the percentage of fragmented and degenerated embryos at the time of first cleavage increase. The lowest proportion of fragmented/degenerated embryos, the highest proportion of two-cell stage embryos, and, consequently, a significantly higher chance of reaching the blastocyst stage are found in those early-cleaving embryos (within 24 – 30 h pi). The early cell divisions of mammalian embryos are characterized for their asynchrony (Han *et al.*, 1999; Somfai *et al.*, 2008; Dang-Nguyen *et al.*, 2010). This phenomenon may be accelerated by polyspermy in pigs (Funahashi, 2003; Somfai *et al.* 2008). Therefore, since the developmental stage and cell number of embryos at a given time point seem to be linked to the time of embryonic genome activation (Minami *et al.*, 2007) and to the rate

of chromosomal abnormalities (Ulloa Ulloa *et al.*, 2008; Dang-Nguyen *et al.*, 2010), selection of embryos with a greater number of blastomeres at the presumed time of first cleavage may rather result in the selection of zygotes with abnormal nuclear status and reduced developmental potential (Booth *et al.*, 2007; Dang-Nguyen *et al.*, 2010; Jeon *et al.*, 2011).

On the other hand, the mechanisms responsible for the findings in **PAPER III** regarding the better chance of those zygotes which cleave earlier after insemination to form blastocysts than their later-cleaving counterparts remains elusive. However, citoplasmatic maturity (Albertini *et al.*, 2003), chromosomal abnormalities (Hardarson *et al.*, 2001) secondary to polyspermy (Funahashi, 2003) and the polyadenylation status of several mRNA transcripts in the oocyte (Brevini *et al.*, 2002) seem to be involved. For instance, Brevini *et al.* (2002) suggested that developmental competence is accompanied by altered amounts of specific maternal mRNA molecules as well as by their abnormal polyadenylation levels with synchrony between polyadenylation and cleavage emerging as an apparently important factor. Although these phenomenons appear to be largely due to an oocyte-effect, it may also have other causes. Indeed, 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 in bovine (Ward *et al.*, 2001). The mechanism by 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.*, 2000). However, from the results in **PAPER III** using semen from the same boar, it is suggested that the kinetics of zygote cleavage and their influence in subsequent developmental ability may also be influenced by the culture condition or by a specific genetic factor, such as the preimplantation embryo development gene already reported in mice and cattle (Lechniak *et al.*, 2008).

Related to this, it is worth noting that regarding the culture conditions, **PAPER III** also reports, for the first time, that the main energy substrate presents in the IVC medium has a significant effect on several developmental parameters among embryos that first cleave at different time points (24, 26, 30 and 48 h pi). First, and as it has been previously mentioned, culture with glucose during the first 48 h pi decreases the kinetics of the IVP embryo development. While the development of almost all embryos cultured with pyruvate and lactate was almost completed by 144 h pi, development continued to progress to the blastocyst stage between 144 and 168 h pi for embryos cultured with glucose, especially for those first cleaved by 24 h pi and between 26 and 30 h pi. However, those embryos that reach the blastocyst stage between 144 and 168 h pi arrested at the early blastocyst stage. Second, the blastocyst development stage and the average number of cells per blastocyst between early- and late-cleaving embryos

differ significantly among embryos cultured in pyruvate- and lactate-based medium, but not in those cultured with glucose. Indeed, the proportions of expanded and early blastocysts were, respectively, lower and higher in those late-cleaving embryos than their earlier counterparts. Similarly, the number of average cells per blastocyst was also lower in those late-cleaving embryos. These differences were not observed in **PAPER II**, when embryos were evaluated regardless of the timing of their first embryonic cleavage.

Considering all these outcomes, findings in **PAPER III** prove that late-cleaving embryos, regardless of the main energy source present in the culture media during the first 48 h of culture, have a significantly lower chance of reaching the blastocyst stage than the early-cleaving embryos. Furthermore, results in both **PAPER II** and **PAPER III** demonstrate that culture medium with pyruvate and lactate reduces embryo development failure and affects the kinetics of embryo development, as it enhances the percentage of blastocysts reaching the most advanced stage. The conditions provided by pyruvate and lactate during the early stages of embryo development also impact the timing of first zygotic cleavage and the ability to overcome the four- or eight-cell block. Therefore, these results suggest that there is a relationship between the better conditions provided by pyruvate and lactate during the early development and the timing of the first embryonic cleavage. As Dang-Nguyen *et al*. (2010) pointed out, porcine embryos cultured in pyruvate and lactate that undergo the first cleavage earlier have a greater chance of developing up to the eight-cell stage within the first 48 h pi. These embryos are more likely to continue to develop if transferred to *in vitro* culture with glucose afterwards, since glucose is a better energy substrate than pyruvate-lactate for development beyond the eight-cell stage. In contrast, the adverse conditions of glucose at very early stages of development lead to arrest a higher number of embryos at the four- or eight cell stage, regardless of when their first cleavage has occurred.

So far, the findings in **PAPER II** and **PAPER III** have shown that culture conditions, such as the main energy substrate present in the IVC media during the first 48 h of culture and the addition of HA, as well as the timing of several events during the earliest stages of development – particularly the timing of the first embryonic cleavage –, appear to be linked themselves and are related to subsequent embryonic competence.

On the other hand, IVC conditions also seem to induce sex-related differences in the kinetics of embryo development, survival, and response to environmental conditions in

a stage-dependent manner, as soon as the first cleavage occurs (Kochhar *et al*., 2001; Bermejo-Àlvarez *et al*., 2008b). Therefore, the availability of optimal IVP systems capable to obtain large number of good-quality embryos, and efficient sex determination techniques to determine the sex of embryos at any developmental stage are truly essential. They let researchers study the sex dimorphism in epigenetic, regulatory, and metabolic pathways and, consequently, their sex-dependent differences in porcine embryo development physiology and *in vitro* survival. In this regard, it is important to establish culture systems capable to skew the sex ratio of the resultant embryos for both porcine breeding programs and basic research using porcine models when necessary.

The first requirement to evaluate the impact of IVC conditions on sex ratio is the design of an appropriate tool. For this reason, **PAPER I** describes a rapid, accurate and reproducible duplex PCR-based method developed to be used for porcine embryo sex determination at various developmental stages, with a high degree of efficiency. This method differs from the few others published in the porcine because it is the first one based on the amplification of repetitive sequences, and it is sensitive enough to amplify the DNA contained in a single diploid cell. In particular, the present duplex PCR-based sexing method was designated to co-amplify the porcine Y chromosome-specific repeat sequence *X12696*[4] (McGraw *et al*., 1988), and the porcine multicopy 12S rRNA mitochondrial gene. The presence or absence of the former determines samples to be male or female, respectively. In contrast, the presence of the latter, common to both sexes, distinguishes amplification failures or female embryos when Y-specific product is absent. Moreover, it is based on the amplification of repetitive sequences, rather than single or low copy ones, since it provides the capacity to amplify small amounts of DNA template with an improved sensitivity, even using a single round of PCR. Thus, the risk of contamination and amplification failure, as well as the overall time required for analysis is reduced.

To establish the optimal conditions, the sensitivity and the specificity of the duplex PCR technique developed in **PAPER I**, different primer sets targeting the *X12696*[5] Y chromosome-specific repeat sequence (*SUSYa* and *SUSYb*; sex-related primer sets), the multicopy porcine-specific mitochondrial 12S rRNA gene (*SUS12S*; control primer set) and the *X51555*[6] *1* chromosome repeat sequence (*SUS1*; control primer set) were designed and their specificity was established. Subsequently, the technique was optimised by testing combinations of two specific primer sets (*SUSYa/SUS12S*; *SUSYb/SUS12S*), different primer concentrations, two sources of DNA polymerase, different melting temperatures, and different numbers of amplification cycles using

[4] See footnote (1)
[5] See footnote (1)
[6] See footnote (1)

genomic DNA from porcine ovarian and testicular tissues before being applied directly to IVP porcine embryos.

Due to the non-specific amplification at all the melting temperatures tested in both male and female genomic DNA for the primer set *SUS1*, the amplification of the *X51555*[7] 1 chromosome repeat sequence as an internal control was discarded. In contrast, the primer set targeting the porcine mitochondrial gene encoding for 12S rRNA was selected as an internal control since it showed a specific amplification in both male and female genomic DNA when the melting temperatures were 55.6ºC, 57.8ºC and 59.8ºC. In addition to the successful amplification conditions provided by the *SUS12S* primer set, the amplification of this sequence is useful for avoiding cross-linking contaminations and for increasing the specificity of the technique since it is one of the most widely used species-specific genetic marker (Prakash *et al*., 2000; Wang *et al*., 2010). On the other hand, two primer sets, *SUSYa* and *SUSYb*, were designed on the *X12696*[8] porcine male-specific repeat sequence and all tested conditions were verified for both primer sets. This allowed us to establish which fitted better and, thus, to provide further support for the correct sex identification. The primer sets were designed on this sequence because it is one of the few porcine Y-specific repeat sequences that have already been sequenced from the porcine genome.

The optimised *SUSYa/SUS12S*- and *SUSYb/SUS12S*-based duplex PCR procedures[9] were first used with genomic DNA (gDNA) obtained from testicular and ovarian domestic pig tissue and, then, used with gDNA from oocytes, and IVP porcine cell-stage embryos and blastocysts. The *SUSYb/SUS12S* procedure was slightly more sensitive than the *SUSYa/SUS12S*, hence *SUSYb/SUS12S* primer-based PCR was the one used in the experiments of **PAPER II** and **PAPER III**. It was sensitive enough (0.001 ng DNA) to identify the sex of porcine single cells and IVP cell-stage embryos (2-, 4-, 8- and 16-cell embryos) with 100% efficiency, as well as the sex of blastocysts with 96.6% accuracy and 96.7% efficiency. The high efficiency of the described embryo sexing method is due, as mentioned above, to its duplex nature based on the amplification of repetitive sequences. Moreover, the high efficiency and sensitivity obtained when embryos and oocytes were sexed is also due to the embryo DNA extraction method and the complete ZP-removal before DNA release. The current PCR technique was performed directly on lysates, avoiding DNA extraction procedures (Leoni *et al*., 2000; Manna *et al*., 2003). Specifically, we combined snap-freezing in liquid nitrogen and sample digestion with proteinase K solution (Bermejo-Àlvarez *et al*., 2008). Proteinase K

[7] See footnote (1)
[8] See footnote (1)
[9] The optimised conditions are shown in Chapter III, page 84 (Table 2)

inactivates the nucleases simultaneously with DNA release, preventing DNA degradation (Mara *et al.*, 2004). The complete removal of ZP and the extensive washing of ZP-free embryos are essential steps because they prevent the presence of accessory spermatozoa attached to the ZP, which may alter the sex-determining results (Greenlee *et al.*, 1998; Bermejo-Alvarez *et al.*, 2008a).

Therefore, the new duplex PCR procedure developed in **PAPER I** can be used for sex diagnosis of small amounts of genomic DNA obtained from different sources, including porcine tissues, embryos at different developmental stages and sex-known single cells (oocytes). For this reason, this technique was used in **PAPER II** and **PAPER III** to determine sexual dimorphisms in survival and quality of IVP porcine cleavage-stage embryos and blastocysts, under specific culture conditions. However, in addition to the use of this method for the sex determination of entire embryos described in the present thesis, this test can have other applications. Indeed, and given its high sensitivity, this method can be used for the sex determination of biopsied blastomeres. Although embryo biopsy is currently not a widespread technology in swine embryo transfer industry (Lopes *et al.*, 2001), sex determination by embryo biopsy and subsequent biopsied embryo culture and transfer are the only reliable embryo sex determination method to obtain offspring of a determined sex. Thus, due to the potential applications of embryo biopsy, not only in sex determination, but also in the identification of genetic markers linked to economic traits, mitochondrial genome analysis and paternity identification, the availability of successful embryo biopsy procedures, as well as PCR techniques like that developed in **PAPER I** are required in the near future (Abolfazl *et al.*, 2010). Furthermore, apart from the application of the described method in reproductive biology, due to its high specie-specificity, this test can be useful in several other sectors including forensics, archaeozoology, and meat production and processing. For instance, it can be used for the detection and quantification of porcine DNA in food and feedstuffs.

Preimplantation sexual dimorphism studies provide a physiological basis for the different survival rates of male and female embryos observed under different conditions, which are responsible for the sex ratio skews occurring after conception and linked to a reduction in fertility. Sex-related physiological differences observed under *in vitro* conditions, such as speed of development, survival after vitrification and cell number, have a molecular root. Thus, the study of this phenomenon in early stages of development will help to understand several 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 the embryonic origin of adult diseases (Bermejo-Alvarez, 2010). At a molecular level, only few evidences, based on the transcription of some specific genes and glucose metabolism, have been reported in bovine, human and murine embryos (Bermejo-Alvarez, 2010), but not in porcine. In preimplantation bovine and human embryos, total glucose metabolism is twice as high in male embryos as in female ones, and the activity of PPP is four times greater in female than in male blastocysts (Tiffin *et al*., 1991; Ray *et al*., 1995). 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 (Mak *et al*., 2004; Okamoto *et al*., 2004; Bermejo-Alvarez *et al*., 2008b). These differences are present at least in bovine (Gutiérrez-Adán *et al*., 2000), human (Taylor *et al*., 2001), and murine (Latham *et al*., 2000) embryos. They affect sex chromosomes-linked genes, but also genes located on autosomes such as interferon-T (Larson *et al*., 2001). Moreover, the differences in the transcription of these genes between genders may implicate a new epigenetic process occurring in early embryos and with implications in sex ratio (Pérez-Crespo *et al*., 2005). During the last decade, three new epigenetic processes have been discovered. First, telomeres of Xq chromosomes in early male embryo are around 1,100 bp shorter than in female Xqs (Perner *et al*., 2003); second, there are gender-dependent changes in the CAG repeat length that increase in early embryos (Kovtun *et al*., 2004) indicating that an X-encoded factor suppresses development, or that an X- or Y-linked factor is involved in the gender-linked expression of replication/repair enzymes that play a role at early stages of embryo division; and third, mice male foetuses have larger placentas than females (Ishikawa *et al*., 2003) indicating that there are also placental differences between males and females, which may explain sex differences in implantation.

In the present thesis, two different approaches have been used to determine, for the first time, sexual dimorphisms in terms of selective loss of embryos of a determined sex under specific conditions and depending on certain events during the earliest stages of development. For this purpose, the accurate, sensitive, and specific duplex PCR method developed in **PAPER I** was used. Even though sexual dimorphism and the putative mechanisms that cause these differences have been described in mice and bovine species, no data is available in porcine. Understanding the results of sex ratio skew studies is really complex. The multifarious relations between the different variables that influence the sex of the offspring make the observations and their subsequent hypothesis rather speculative without a molecular or physiological basis. For these reasons, mechanistic studies together with meta-analyses and physiological markers are necessary to find the real meaning of different observations (Bermejo-Alvarez *et al*., 2011). In the current thesis, findings in **PAPER II** and **PAPER III** have reported the first

evidences of different survival rates of porcine male and female embryos under different culture conditions. Although a small number of samples have been studied, the data provided by **PAPER II** and **PAPER III** clearly warrant further research on sex ratio skews in IVP porcine embryos under some IVC conditions. This will definitely contribute to elucidating the molecular mechanisms that would explain and offer more consistency and cohesiveness to our results.

In **PAPER II**, embryos produced in glucose or pyruvate and lactate during the first 48 h pi together with glucose from 48 to 168 h pi, and 0.5 mg·mL^{-1} HA, 1.0 mg·mL^{-1} HA or without HA during all the culture period were sexed at the blastocyst stage. Previous studies in bovine have suggested that fluctuations in the culture environment during the first 48 h pi may cause a preferential loss of female bovine embryos by 120 h pi (Yadav *et al.*, 1993), while others have demonstrated that culture environment does not preferentially support the development of one sex over another since the overall sex ratio at the 2- cell stage and the blastocyst stage is similar (Rizos *et al.*, 2008a). As mentioned above, energy substrates do not alter the effects of HA on embryo development, but do influence the effect of HA on the sex ratio. The decrease in blastocyst formation yield when embryos are cultured with 1.0 mg·mL^{-1} HA in both glucose and pyruvate- and lactate-based media seems to be caused by a preferential loss of female embryos. This appears, in turn, to lead to a slight skew in the sex ratio towards males only in the pyruvate-lactate group of embryos, but by an equal loss of embryos of both sexes in the glucose group. Although the exact mechanism by which pyruvate-lactate, but not glucose supplementation, interacts with HA and leads to these outcomes is still unknown, this interaction must take place at the very early stages of embryo development because the IVC medium was supplemented by either glucose or pyruvate-lactate only during the first 48 h pi. Therefore, findings in **PAPER II** are in agreement with those reported by Yadav *et al.* (1993) and Rizos *et al.* (2008a), and suggest that culture environment from 48 to 168 h pi does not preferentially support the development of one sex over another, but it is the culture environment before 48 h pi which does it. This suggestion is also supported by results from **PAPER III**.

In **PAPER III**, IVP porcine embryos cultured with glucose or pyruvate-lactate were selected and cultured separately according to the time of their first embryonic cleavage (24, 26, 30 and 48 h pi). Afterwards, cleaved embryos (three replicates) and blastocysts (four replicates) were sexed at 48 h pi and 168 h pi, respectively, in order to determine the putative effects of energy substrate and the timing of the first cleavage on the sex ratio of both cleavage-stage embryos and blastocysts. Regardless of the energy substrate, i.e., in both glucose and pyruvate-lactate groups of embryos, **PAPER III** shows a sex ratio deviation towards males among embryos that completed their first

cell cycle before 30 h pi, and towards females among those embryos that undergo the first cleavage later. Although there is no published evidence in porcine embryos, this observation is consistent with previous data on the general predominance of male sex among bovine cleavage-stage embryos cleaved before 30-32 h pi (Avery *et al.*, 1991; Xu *et al.*, 1992; Yadav *et al.*, 1993; Rizos *et al.*, 2008a).

It is known that there are many differences at the transcriptional level between male and female preimplantation embryos. Many genes with gender-dependent expression are located on the X-chromosome (200 genes). However, significant differences in expression have also been found in genes located either on the Y- or on autosomal chromosomes (Gutiérrez-Adán *et al.*, 2006). Thus, it has been hypothesized that differential metabolism and growth rates may be attributable to the unbalanced expression of X-linked genes between the sexes during some stages of the early preimplantation development. Indeed, while the expression of some genes located on the Y-chromosome may accelerate the growth of male preimplantation embryos, the expression of others, located on the paternally derived X-chromosome or expressed on both X-chromosomes, retards development (Epstein *et al.*, 1978; Burgoyne *et al.*, 1995; Mak *et al.*, 2004; Okamoto *et al.*, 2004). For instance, the high levels of *G6PD* have been proposed to account for the delayed development of *in vitro* female embryos by mediating NADPH production and modulating ROS levels (Gutiérrez-Adán *et al* 2000; Pérez-Crespo *et al.*, 2005). Likewise, the increased level of expression of *GLUT1* gene in males is associated with the faster development of male embryos under *in vitro* conditions, since faster development may lead to an increase in glucose uptake mediated by *GLUT1* expression (Avery *et al.*, 1991; Xu *et al.*, 1992). Furthermore, it is known that, in terms of free radical scavenging, males have more H_2O_2 than females and, under normal circumstances, these H_2O_2 levels promote higher cell proliferation in males (Hansen *et al.*, 1999).

Considering that embryos first cleaved before 30 h pi account for most of the subsequent blastocysts in both energy-source groups in **PAPER III**, it is reasonable to predict a skew on the sex ratio towards males of blastocysts first cleaved at this recorded time. However, in the context of our study, the sex ratio deviation at the blastocyst stage was only noted within embryos cultured with pyruvate and lactate. These results, as those in **PAPER II**, suggest that some differences between male and female embryos are detectable before the major burst of gene activation, and they are only manifested under some environmental conditions. Indeed, the energy substrate present in the medium during the first 48 h of culture seems to have a differential and critical effect on the selective loss of embryos of one determined sex, and will thereby modify the sex ratio of embryos that reach the blastocyst stage. The

suboptimal conditions associated with glucose in the culture medium during the first 48 h pi may lead to a selective loss of male embryos first cleaved at 30 h pi, resulting in a similar proportion of male and female blastocysts at 168 h pi.

Embryos acquire the ability to respond to environmental stress caused by culture conditions as development progresses (Edwards *et al.,* 2001; Bermejo-Alvarez *et al*., 2011). There is some evidence suggesting that the sex of the embryo may play a role in the ability of the embryo to mount a stress response during early stages of development. In humans, embryo transfer at early stages does not skew the sex ratio (Lansac *et al.,* 1997), whereas several reports suggest a sex ratio skew in the offspring when embryos are transferred at the blastocyst stage (Tarin *et al.,* 1995, Quintans *et al.,* 1998, Ménézo *et al.,* 1999). Moreover, it has been reported that human and bovine female preimplantation embryos resist oxidative stress conditions better than male embryos (Gutiérrez-Adán *et al*., 2000; Taylor *et al*., 2001; Iwata *et al*., 2002; Wrenzycki *et al*., 2002). One source of this better resistance seems to be the higher expression of G6PD by those female embryos. Glucose-6-phosphate dehydrogenase is the only NADPH-producing enzyme activated in response to oxidative stress (Filosa *et al*., 2003) and it plays a crucial role in the detoxification of H_2O_2 and in the protection from redox-stress induced apoptosis (Fico *et al*., 2004). Consistently, it has been proven that G6PD inhibition removes some of the sexual dimorphism in mice (Pérez-Crespo *et al*., 2005) and bovine (Kimura *et al*., 2005). The fact that stress favours female embryos *in vitro* is consistent with the sex allocation hypothesis of Trivers and Willard (1973). This sex ratio theory predicts that natural selection in mammals should favour an excess of male offspring only when mothers are in good survival conditions, whereas endangered mothers would benefit by producing daughters. Thus, it is conceivable that parents could adjust the sex of their offspring in response to environmental conditions (Pérez-Crespo *et al*., 2005). Conversely, the presence of high concentrations of glucose during IVC seems to inhibit the development into blastocyst of female embryos more than that of the male counterparts (Gutiérrez-Adán *et al*., 2001), indicating a selective toxicity towards female embryos. This may be due to a greater flux of the hexose in female embryos through the PPP that, in turn, may determine the abnormal production of free radicals (Kimura *et al*., 2005; Bermejo-Alvarez *et al*., 2008b). Moreover, excess of hypoxanthine phosphoribosyl transferase (HPRT) expression, together with the enhanced flux of glucose through the PPP, could also contribute to purine nucleotide imbalance in female embryos (Rubessa *et al*., 2011). In addition, a later report has demonstrated that female embryos show a slower developmental rate and are more sensitive to cryopreservation (Nedambale *et al*., 2004). Likewise, whereas King *et al*. (1992) concluded that exposure to temperature fluctuation during culture, cryopreservation and transfer resulted in a loss of female embryos, others reported that

significantly more female blastocysts were observed on Day 9 after exposure to transient elevated temperatures on Day 3 of development (Kawarsky, 1999; Edwards *et al*., 2001). Therefore, response to different environmental stresses may be influenced by both stage of development and sex chromosome composition (Kochhar *et al*., 2001).

Although very little is known about the way in which early exposure to glucose or pyruvate and lactate causes a selective loss of a specific sex porcine embryos under different *in vitro* conditions, findings from both **PAPER II** and **PAPER III** support the theory that the source of energy substrate in the medium influences development in a sex related manner as Kochhar *et al*. (2001) already proposed.

Indeed, according to the outcomes and conditions in **PAPER II**, replacing glucose with pyruvate and lactate at the early stages of IVP provides the porcine embryos with more suitable conditions for cellular oxidation-reduction equilibrium, higher oxygen consumption and ATP demand to accommodate increased protein synthesis and activity of the Na^+/K^+-dependent ATPase (Lopes *et al*., 2007), resulting in higher embryo development. On the other hand, embryos cultured with HA have been reported to have less total ATP content due to impaired enzymatic conversion of ATP due to reduced adenylate cyclase activity (Dittman *et al*., 1998; Stojkovic *et al*., 2002). Moreover, it is known that oxygen consumption tends to be higher in good-quality female bovine embryos than in male embryos (Lopes *et al*., 2007). Therefore, both the higher oxygen consumption and the higher potential lack of ATP may cause additional stressful conditions in those female embryos cultured in IVC medium with pyruvate and lactate and 1.0 $mg \cdot ml^{-1}$ HA, resulting in a tendency for a sex ratio bias towards males.

Alternatively, the mechanism by which early exposure to glucose (not to pyruvate and lactate) causes a selective loss of male embryos first cleaved at 30 h pi at later developmental stages in **PAPER III** could be related to the adaptive response to the detrimental effects of oxidative stress associated with glucose metabolism. As aforementioned, the presence of glucose at early stages of development provides the porcine embryos with worse suitable conditions for cellular oxidation-reduction balance. Moreover, it is assumed that shorter intervals between cell divisions increase cells vulnerability to toxic insult (Harvey *et al*., 2002) and, thus, a more rapid growth of the male embryos increases susceptibility to lethal changes that, together with their higher sensitivity to oxidative damage, may ultimately affect the sex ratio towards females.

This thesis provides new knowledge about physiology, development and sexual dimorphisms in survival rates and kinetics of the development of IVP porcine embryos under specific *in vitro* culture conditions. Furthermore, this work develops a new suitable procedure for determining the sex of porcine embryos at different developmental stages, and establishes new conditions that may enhance porcine IVP techniques. Culture conditions, e.g. presence of optimal concentrations of specific energy substrates and HA at the appropriate time of embryo development, as well as the timing of several events during the earliest stages of development, particularly the timing of the first embryonic cleavage, appear to be linked amongst themselves and are related to subsequent embryonic competence. Moreover, the use of the new duplex PCR technique, developed in this thesis, reveals a sex ratio deviation among embryos first cleaved at different time intervals. Likewise, it provides evidence that the source of energy substrate in culture medium influences development in a sex related manner. Therefore, all findings presented herein may help to better understand the role of specific *in vitro* conditions in regulating the key events in porcine embryo development and may, thus, allow designing new IVC protocols. Moreover, results about selective loss of porcine male or female embryos under these specific *in vitro* conditions highlights novel physiological markers which clearly warrant further research towards the molecular mechanisms responsible for those sex ratio skews, and provides the basis of how to enhance *in vitro* production of porcine embryos in a sex-related manner.

Chapter 7

General Conclusions

1. The new developed duplex PCR procedure, based on the amplification of the porcine Y chromosome-specific repeat sequence *X12696*[10] and the porcine multicopy 12S rRNA mitochondrial gene, is highly reliable, specific and sensitive for sexing porcine tissues, IVP embryos at different developmental stages and oocytes.

2. Pyruvate and lactate at specific concentrations of 0.17 and 2.73 mM, respectively, are the preferred energy substrates at the early stages of porcine embryo development in comparison to 5.55 mM glucose. Culture with pyruvate and lactate reduces embryo development failure and affects the kinetics of embryo development as it enhances the percentage of blastocysts reaching more advanced stages.

3. Under the experimental conditions used in this thesis, the effects of HA on porcine embryo development depend on HA concentration and are not influenced by the main energy substrate present in the culture medium during the first 48 h pi. A concentration of 0.5 mg·ml^{-1} HA has no effect on embryo development, whereas 1.0 mg·ml^{-1} HA significantly decreases blastocyst rates on both glucose- and pyruvate and lactate-based media.

4. The successful development to the blastocyst stage markedly decreases with an increase in both the time from fertilisation to first embryonic cleavage and the percentage of fragmented and degenerated embryos at the time of first cleavage.

5. The development of embryos that first cleaved from 24 to 30 h pi proceeds more successfully as a blastocyst population than late-cleaving ones (within 30-48 h pi). The highest chance of developing to blastocysts is for embryos cleaved

[10] See footnote (1)

by 24 h pi and the uppermost percentage of cleaved embryos is obtained at 30 h pi.

6. The energy substrate present in the culture medium during the first 48 h of culture has a differential influence on the blastocyst stage and the average number of cells per blastocyst between early- and late-cleaving embryos. Culture with pyruvate and lactate, but not with glucose, causes a detrimental effect on late-cleaving embryos (30-48 h pi), increasing the proportion of early blastocysts with fewer average cells.

7. There is a predominance of male sex among porcine cleavage-stage embryos cleaved within 26 and 30 h pi, and a predominance of female sex among those cleaved within 30 and 48 h pi, in both energy source groups.

8. The energy substrate present in the medium during the first 48 h of culture has a differential effect on the selective loss of embryos of one determined sex. Culture with glucose leads to a selective loss of male embryos first cleaved between 26 and 30 h pi, resulting in a similar proportion of male and female blastocysts at 168 h pi. In contrast, culture with pyruvate and lactate results in an equal loss of both sexes, maintaining the sex ratio deviation towards males among cleavage-stage embryos and blastocysts.

9. The energy substrate present in the culture medium during the first 48 h pi influences the effect of HA on the sex ratio of embryos. The decrease in blastocyst rate in those embryos cultured with 1.0 $mg \cdot ml^{-1}$ HA seems to be caused by a preferential loss of female embryos, leading to a slight skew in the sex ratio towards males in the group of embryos cultured with cultured with pyruvate and lactate, but by an equal loss of embryos of both sexes in the glucose group.

References

Abeydeera LR, Johnson LA, Welch GR, Wang WH, Boquest AC, Cantley TC, Rieke A, Day BN. 1998. *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 50:981-988.

Abeydeera LR, Prather RS, Day BN. 1999. *Developmental ability of in vitro-derived pig embryos in four different media*. Biol Reprod Suppl 60:128 (Abstract).

Abeydeera LR. 2002. *In vitro production of embryos in swine.* Theriogenology 57(1):256-273.

Abolfazl S, Sara B, Hassan N, Ebrahim A, Banafsheh H, Amin B. 2010. *Effects of timing of cell biopsy from pre-compacted morula stage bovine embryos on subsequent embryonic development.* J Reprod Infertil 11(1):25-32.

Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, 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.

Akane A, Seki S, Shiono H, Nakamura H, Hasegawa M, Kagawa M, Matsubara K, Nakahori Y, Nagafuchi S, Nakagome Y. 1992. *Sex determination of forensic samples by dual PCR amplification of an X-Y homologous gene*. Forensic Sci Int 52(2):143-148.

Allen RL, Wright RW Jr. 1984. *In vitro development of porcine embryos in coculture with endometrial cell monolayers or culture supernatants*. J Anim Sci 59(6):1657-1661.

Almiñana C, Gil MA, Cuello C, Caballero I, Roca J, Vazquez JM, Martinez EA. 2008. *In vitro fertilization (IVF) in straws and short gamete coincubation time improves the efficiency of porcine IVF*. Reprod Dom Anim 43:747-752.

Alomar M, Tasiaux H, Remacle S, George H, Paul D, Donnay I. 2008. *Kinetics of fertilization and development, and sex ratio of bovine embryos produced using the semen of different bulls.* Anim Reprod Sci 107(1-2):48-61.

Albertini DF, Sanfins A, Combelles CMH. 2003. *Origins and manifestations of oocyte maturation competencies.* RBM Online 6:410-415.

Anbari K, Schultz RM. 1993. *Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos in vitro.* Mol Reprod Dev 35(1):24-28.

Appa Rao KB, Totey SM. 1999. *Cloning and sequencing of buffalo male-specific repetitive DNA: sexing of in-vitro developed buffalo embryos using multiplex and nested polymerase chain reaction*. Theriogenology 51(4):785-797.

Avery B, Madison V, Schmidt M. 1991. *Sex and development in bovine in vitro-fertilized embryos*. Theriogenology 35:953–963.

Avery B, Jørgensen CB, Madison V, Greve T. 1992. *Morphological development and sex of bovine in vitro-fertilized embryos*. Mol Reprod Dev 32(3):265-270.

Baltz JM. 2012. *Media composition: salts and osmolality.* Methods Mol Biol 912:61-80.

Bannai S, Ishii T. 1988. *A novel function of glutamine in cell culture: utilization of glutamine for the uptake of cystine in human fibroblasts.* J Cell Physiol 137(2):360-366.

Barnett DK, Clayton MK, Kimura J, Bavister BD. 1997. *Glucose and phosphate toxicity in hamster preimplantation embryos involves disruption of cellular organization, including distribution of active mitochondria*. Mol Reprod Dev 48:227–237.

Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, Avendaño C, Oehninger S. 2009. *Developmental sperm contributions: fertilization and beyond.* Fertil Steril 92:835-848.

Bavister BD. 1995. *Culture of preimplantation embryos: facts and artifacts*. Hum Reprod Update 1:91-148.

Beckmann LS, Day BN. 1993. *Effect of médium NaCl concentration and osmolarity ion cultura of the early stage porcine embryo and viability of embryos cultured in a selected superior médium*. Theriogenology 39:611-622.

Beebe LF, McIlfactrick S, Nottle MB. 2007. *The effect of energy substrate concentration and amino acids on the in vitro development of preimplantation porcine embryos*. Cloning Stem Cells 9:206–215.

Beebe LF, Vassiliev I, McIlfatrick S, Nottle MB. 2009. *Adding essential amino acids at a low concentration improves the development of in vitro fertilized porcine embryos.* J Reprod Dev 55(4):373-377.

Beebe LF, McIlfatrick SM, Vassiliev IM, Nottle MB. 2013. *Development of an improved porcine embryo culture medium for cloning, transgenesis and embryonic stem cell isolation.* Clon Transgen 2:107.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. 2008a. *Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially?* Biol Reprod 79(4):594-597.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. 2008b. *Epigenetic differences between male and female bovine blastocysts produced in vitro.* Physiol Genomics 32(2):264-272.

Bermejo-Alvarez P. 2010. *Sex ratio control before fertilization and preimplantational sexual dimorphism in bovine*. Doctoral thesis, Computense University of Madrid. Available on-line via TDX home: <http://tdx.cat/handle/10803/48106> [Accessed: April 10, 2013]

Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A. 2010a. *Transcriptional sexual dimorphism in elongating bovine embryos: implications for XCI and sex determination genes*. Reproduction 141(6):801-808.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. 2010b. *Sex determines the expression level of one-third of the actively expressed genes in bovine blastocysts*. Proc Natl Acad Sci USA 107:3394–3399.

Bermejo-Alvarez P, Rizos D, Lonergan P, Guiterrez-Adan A. 2011. *Transcriptional sexual dimporphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease.* Reproduction 141(5):563-570.

Bermejo-Alvarez P, Roberts Rm, Rosenfeld CS. 2012. *Effect of glucose concentration during in vitro culture of mouse embryos on development to blastocyst, success of embryo transfer, and litter sex ratio.* Mol Reprod Dev 79(5):329-336.

Bernardi ML, Delouis C. 1996. *Sex-related differences in the developmental rate of in-vitro matured/in-vitro fertilized ovine embryos*. Hum Reprod 11(3):621-626.

Betts DH, King WA. 2001. *Genetic regulation of embryo death and senescence*. Theriogenology 55:171–191.

Biggers JD, Whittingham DG, Donahue RP. 1967. *The pattern of energy metabolism in the mouse oocyte and zygote*. Proc Natl Acad Sci USA 58:560–567.

Biggers JD, Lawitts JA, Lechene CP. 1993. *The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro.* Mol Reprod Dev 34(4):380-390.

Bird E, Contreras R. 1986. *Maternal dietary sodium chloride levels affect the sex ratio in rat litters*. Physiol Behav 36:307–310.

Block J, Drost M, Monson RL, Rutledge JJ, Rivera RM, Paula-Lopes FF, Ocon OM, Krininger CE 3rd, Liu J, Hansen PJ. 2003. *Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed, lactating cows*. J Anim Sci 81(6):1590-1602.

Block J, Bonilla L, Hansen PJ. 2009. *Effect of addition of hyaluronan to embryo culture medium on survival of bovine embryos in vitro following vitrification and establishment of pregnancy after transfer to recipients*. Theriogenology 71:1063–1071.

Bodmer M, Janett F, Hässig M, den Daas N, Reichert P, Thun R. 2005. *Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions*. Theriogenology 64(7):1647-1655.

Bonet S, Briz M, Pinart E, Sancho S, García-Gil N, Badia E. 2000. *Morphology of Boar Spermatozoa. Morfologia Espermàtica en Porcí. Morfología Espermática en Porcino*. Ed. Institut d'Estudis Catalans. Barcelona.

Booth PJ, Holm P, Callesen H. 2005a. *The effect of oxygen tension on porcine embryonic development is dependent on embryo type*. Theriogenology 63(7):2040-2052.

Booth PJ, Humpherson PG, Watson TJ, Leese HJ. 2005b. *Amino acid depletion and appearance during porcine preimplantation embryo development in vitro*. Reprod 130:655–668.

Booth PJ, Watson TJ, Leese HJ. 2007. *Prediction of porcine blastocyst formation using morphological, kinetic, and amino acid depletion and appearance criteria determined during the early cleavage of in vitro-produced embryos*. Biol Reprod 77:765-779.

Borg N, Holland M. 2008. *The effect of glycosaminoglycans on rat gametes in vitro and the associated signal pathway*. Reproduction 135(3):311-319.

Bormann C, Swain J, Ni Q, Kennedy R, Smith G. 2006. *Preimplantation embryo secretome identification*. Fertil Steril (Suppl. 2) 86:116.

Bos-Mikich A, Mattos AL, Ferrari AN. 2001. *Early cleavage of human embryos: an effective method for predicting successful IVF/ICSI outcome*. Hum Reprod 16:2658–2661.

Bredbacka P. 2001. *Progress on methods of gene detection in preimplantation embryos*. Theriogenology 55(1):23-34.

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage and its relation with developmental competence. Mol Reprod Dev 63(4):510-517.

Bryla M, Trzcinska M, Wieczorek J. 2009. *Analysis of in vivo- and in vitro derived pig expanded blastocysts based on DNA fragmentation.* Anim Sci Pap Rep 27:59-68.

Buettner GR. 1993. *The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate*. Arch Biochem Biophys 300(2):535-543.

Burgoyne PS, Thornhill AR, Boudrean SK, Darling SM, Bishop CE, Evans EP. 1995. *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.

Bussalleu E, Torner E. 2013. *Quality improvement of boar seminal doses.* In: Bonet S, Casas I, Holt WV, Yeste M Eds. Boar Reproduction: Fundamentals and New Biotechnological Trends. Ed. Springer: Berlin, Germany. pp 517-550. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8.

Caamaño JN, Ryoo ZY, Thomas JA, Youngs CR. 1996. *Beta-mercaptoethanol enhances blastocyst formation rate of bovine in vitro-matured/in vitro-fertilized embryos*. Biol Reprod 55:1179–1184.

Cameron EZ. 2004. *Facultative adjustment of mammalian sex ratios in support of the Trivers-Willard hypothesis: evidence for a mechanism*. Proc Biol Sci 271:1723–1728.

Cameron RD, Beebe LF, Blackshaw AW. 2006. *Cryopreservation and transfer of pig embryos.* Soc Reprod Fertil Suppl 62:277-291.

Campbell S, Swann HR, Aplin JD, Seif MW, Kimber SJ, Elstein M. 1995. *CD44 is expressed throughout preimplantation human embryo development*. Hum Reprod 10:425–430.

Carneiro MC, Takeuchi PL, Araújo A, Lôbo RB, Elias FP, Vila RA, Miranda-Furtado CL, Ramos ES. 2011. *Sexing single bovine blastomeres using TSPY gene amplification*. Genet Mol Res 10(4):3937-3941.

Casas I. 2010. *A practical approach on boar sperm cryodamage. Morphofunctional and immunocytochemical study of cryopreserved boar sperm intented for use in artificial insemination.* Doctoral thesis, University of Girona.
Available on-line via TDX home: <http://www.tdx.cat/handle/10803/7642>
[Accessed: May 7, 2013]

Casas I, Flores E. 2013. *Gene Banking: the Freezing Strategy.* In: Bonet S, Casas I, Holt WV, Yeste M Eds. Boar Reproduction: Fundamentals and New Biotechnological Trends. Ed. Springer: Berlin, Germany. pp 551-588. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8.

Cassar G, de la Fuente R, Yu Z, King GJ, King WA. 1995. *Sex chromosome complement and developmental diversity in pre- and posthatching porcine embryos*. Theriogenology 44:879–884.

Castillo-Martín M, Yeste M, Morató R, Mogas T, Bonet S. 2013a. *Cryotolerance of in vitro-produced porcine blastocysts is improved when using glucose instead of pyruvate and lactate during the first 2 days of embryo culture*. Reprod Fertil Dev 25(5):737-745.

Castillo-Martín M, Bonet S, Morató R, Yeste M. 2013b. *Comparative effects of adding b-mercaptoethanol or l-ascorbic acid to culture or vitrification warming media on IVF porcine embryos.* Reprod Fertil Dev doi: 10.1071/RD13116 [In press].

Chang HS, Cheng WT, Wu HK, Choo KB. 2000. *Identification of genes expressed in the epithelium of porcine oviduct containing early embryos at various stages of development*. Mol Reprod Dev 56(3):331-335.

Chatot CL, Tasca RJ, Ziomek CA. 1990. *Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium.* J Reprod Fertil 89(1):335-346.

Chong SS, Kristjansson K, Cota J, Handyside AH, Hughes MR. 1993. *Preimplantation prevention of X-linked disease: reliable and rapid sex determination of single*

human cells by restriction analysis of simultaneously amplified ZFX and ZFY sequences. Hum Mol Genet 2(8):1187-1191.

Choudhary M, Zhang X, Stojkovic P, Hyslop L, Anyfantis G, Herbert M, Murdoch AP, Stojkovic M, Lako M. 2007. *Putative role of hyaluronan and its related genes, HAS2 and RHAMM, in human early preimplantation embryogenesis and embryonic stem cell characterization*. Stem Cells 25(12):3045-3057.

Cluzan R, Causeret J, Hugot D. 1965. *Le metabisulfite de potassium. Etude de toxicite a long terme chez le rat*. Ann Biol Anim Biochem Biophys 5:267–281.

Comizzoli P, Marquant-Le Guienne B, Heyman Y, Renard JP. 2000. *Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes*. Biol Reprod 62:1677-1684.

Craig J, Zhu H, Dyce PW, Wen L, Li J. 2005. *Leptin enhances porcine preimplantation embryo development in vitro*. Mol Cell Endocrinol 229:141-147.

d'Apice AJ, Cowan PJ. 2009. *Xenotransplantation: The next generation of engineered animals*. Transpl Immunol 21:111–115.

Dang-Nguyen TQ, Kikuchi K, Somfai T, Ozawa M, Nakai M, Maedomari N, Viet-Linh N, Kanai Y, Nguyen BX, Nagai T. 2010. *Evaluation of developmental competence of in vitro produced porcine embryos based on the timing, pattern and evenness of the first cleavage and onset of the second cleavage*. J Reprod Dev 56:593-600.

Dang-Nguyen TQ, Somfai T, Haraguchi S, Kikuchi K, Tajima A, Kanai Y, Nagai T. 2011. *In vitro production of porcine embryos: current status, future perspectives and alternative applications.* Anim Sci J 82(3):374-382.

Dattena M, Mara L, Bin T AA, Cappai P. 2007. *Lambing rate using vitrified blastocysts is improved by culture with BSA and hyaluronan*. Mol Reprod Dev 74(1):42-47.

Day BN. 2000. *Reproductive biotechnologies: current status in porcine reproduction*. Anim Reprod Sci 60-61:161-172.

de Moraes AA, Paula-Lopes FF, Chegini N, Hansen PJ. 1999. *Localization of granulocyte-macrophage colony-stimulating factor in the bovine reproductive tract*. J Reprod Immunol 42(2):135-145.

Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural (DAAM). Generalitat de Catalunya. 2013. Observatori del porcí. Informe del sector porcí. Exercici 2012.
Availiable on-line via: <http://gsp.iws.es/sites/default/files/u6/Informe%20del%20sector%20porc%C3%AD%20catal%C3%A0%202012.pdf>
[Accessed: January 31, 2014]

Dittmann J, Keller C, Harisch G. 1998. *Inhibition of adenylate cyclise of bat hepatic membranes by glycosaminoglycans*. Life Sci 63:2199–2208.

Dobrinsky JF, Johnson LA, Rath D. 1996. *Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development.* Biol Reprod 72:479-485.

Dominko T, First NL. 1997. *Relationship between maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos*. Theriogenology 47:1041–1050.

Drickamer LC. 1990. *Seasonal variation in fertility, fecundity and litter sex ratio in laboratory and wild stocks of house mice (Mus domesticus)*. Lab Anim Sci 40:284–288.

Du Y, Li J, Kragh PM, Zhang Y, Schmidt M, Bogh IB, Zhang X, Purup S, Kuwayama M, Jorgensen AL, Pedersen AM, Villemoes K, Yang H, Bolund L, Vajta G. 2007. *Piglets*

born from vitrified cloned blastocysts produced with a simplified method of delipation and nuclear transfer. Cloning Stem Cells 9:469–476.

Dumollard R, Carroll J, Duchen MR, Campbell K, Swann K. 2009. *Mitochondrial function and redox state in mammalian embryos*. Semin Cell Dev Biol 20:346–353.

Eagle H. 1959. *Amino acid metabolism in mammalian cell cultures.* Science 130(3373):472-437.

Edwards GL, King WA, Kawarsky S J, Ealy AD. 2001. *Responsiveness of early embryo to environmental insults: potential protective roles for HSP70 and glutathione*. Theriogenolgy 55:209-224.

Edwards RG, Gardner RL. 1967. *Sexing of live rabbit blastocysts*. Nature 214:576–577.

Eid LN, Lorton SP, Parish JJ. 1994. *Paternal influence on S-phase in the first cell cycle of the bovine embryo*. Biol Reprod 51:1232-1237.

Ekser B, Rigotti P, Gridelli B, Cooper DK. 2009. *Xenotransplantation of solid organs in the pig-to-primate model.* Transpl Immunol 21(2):87-92.

El Shourbagy SH, Spikings EC, Freitas M, St John JC. 2006**.** *Mitochondria directly influence fertilisation outcome in the pig*. Reproduction 131:233-245.

Eppig JJ. 1979. *FSH stimulates hyaluronic acid synthesis by oocyte–cumulus cell complexes from mouse preovulatory follicles*. Nature 281:483–484.

Epstein CJ, Smith S, Travis B, Tucker G. 1978. *Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos*. Nature 274:500–503.

European Union. 2010. *Animal welfare: voluntary end to the surgical castration of piglets by 2018*.
Available on-line via European Union: <http://ec.europa.eu/food/animal/welfare/farm/docs/castration_pigs_press_release_en.pdf>
[Accessed: March 4, 2014]

European Union. 2011. Statistical Office of the European Communities (EUROSTAT). *Eurostat Pocketbooks. Food: from farm to fork statistics.*
Available on-line via *Eurostat Home - European Comission*: <http://epp.eurostat.ec.europa.eu/cache/ITY_OFFPUB/KS-32-11-743/EN/KS-32-11-743-EN.PDF>
[Accessed: April 9, 2013]

European Union. 2013. Statistical Office of the European Communities (EUROSTAT). *Eurostat Pocketbooks. Agriculture, forestry and fishery statistics.*
Available on-line via *Eurostat Home - European Comission*: <http://epp.eurostat.ec.europa.eu/portal/page/portal/product_details/publication?p_product_code=KS-FK-13-001>
[Accessed: February 2, 2014]

Fàbrega A. 2012. *A molecular approach on sperm changes during epididymal maturation, ejaculation and in vitro capacitation of boat spermatozoa.* Doctoral thesis, University of Girona.
Available on-line via TDX home: <http://www.tdx.cat/handle/10803/84064>
[Accessed: January 10; 2014]

Fahning ML, Schultz RH, Graham EF. 1967. *The free amino acid content of uterine fluids and blood serum in the cow.* J Reprod Fertil 13(2):229-236.

Fenwick J, Platteau P, Murdoch AP, Herbert M. 2002. *Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro*. Hum Reprod 17:407–412.

Feugang JM, de Roover R, Moen A, Léonard S, Dessy F, Donnay I. 2004. *Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents*. Theriogenology 61:71–90.

Fico A, Paglialunga F, Cigliano L, Abrescia P, Verde P, Martini G, Laccarino I, Filosa S. 2004. *Glucose-6-phosphate dehydrogenase plays a crucial role in protection from redox-stress-induced apoptosis*. Cell Death Differ 11:823–831.

Filosa S, Fico A, Paglialunga F, Balestrieri M, Crooke A, Verde P, Abrescia P, Bautista JM,MartiniG. 2003. *Failure to increase glucose consumption through the pentose-phosphate pathway results in the death of glucose-6-phosphate dehydrogenase gene-deleted mouse embryonic stem cells subjected to oxidative stress*. Biochem J 370:935–943.

Fisher B, Bavister BD. 1993. *Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits*. J Reprod Fertil 99:673-679.

Flisikowska T, Kind A, Schnieke A. 2013. *Genetically modified pigs to model human diseases.* J Appl Genet [In press].

Flood MR, Weibold JL. 1988. *Glucose metabolism by preimplantation pig embryos*. J Reprod Fertil 84:7–12.

Fontanesi L, Scotti E, Russo V. 2008. *Differences of the porcine amelogenin X and Y chromosome genes (AMELX and AMELY) and their application for sex determination in pigs*. Mol Reprod Dev 75:1662–1668.

Food and Agriculture Organization of the United Nations (FAOSTAT). February 2013. Available on-line via *Statistics Division of the Food and Agriculture Organization of the United Nations*: <http://faostat.fao.org/site/339/default.aspx>
[Accessed: February 3, 2014]

Fraser RB, Waite SL, Wood KA, Martin KL. 2007. *Impact of hyperglycemia on early embryo development and embryopathy: In vitro experiments using a mouse model*. Hum Reprod 22:3059–3068.

Fricke PM. 2002. *Scanning the future--ultrasonography as a reproductive management tool for dairy cattle*. J Dairy Sci 85(8):1918-1926.

Frijters AC, Mullaart E, Roelofs RM, van Hoorne RP, Moreno JF, Moreno O, Merton JS. 2013. *What affects fertility of sexed bull semen more, low sperm dosage or the sorting process?* Theriogenology 71(1):64-67.

Fu Q, Zhang M, Qin WS, Lu YQ, Zheng HY, Meng B, Lu SS, Lu KH. 2007. *Cloning the swamp buffalo SRY gene for embryo sexing with multiplex-nested PCR*. Theriogenology 68(9):1211-1218.

Fukuda A, Noda Y, Tsukui S, Matsumoto H, Yano J, Mori T. 1987. *Influence of wáter on in vitro fertilization and embryo development for the mouse*. J In Vitro Fert Embryo Tranf 4(1):40-45.

Funahashi H, Kim NH, Stumpf TT, Cantley TC, Day BN. 1996. *Presence of organic osmolytes in maturation medium enhances cytoplasmatic maturation porcine oocytes.* Biol Reprod 54:1412-1419.

Funahashi H, Cantley TC, Day BN. 1997. *Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization.* Biol Reprod 57:49-53.

Funahashi H. 2003. *Polyspermic penetration in porcine IVM-IVF systems.* Reprod Fertil Dev 15:167-177.

Furnus CC, Valcarcel A, Dulout FN, Errecalde AL. 2003. *The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos*. Theriogenology 60(9):1633-1644.

Gajda B, Smorag Z. 2004. *Cell number in pig blastocysts cultured in different media*. Ann Anim Sci 4:315-320.

Gajda B, Bryla M, Smorag Z. 2008. *Effects of protein source, vitamin E and phenazine ethosulfate on developmental competence ans quality of porcine embryos cultured in vitro.* Folia Biol (Krakow) 58(1-2):57-63.

Gajda B. 2009. *Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology.* Reprod Biol 9:97-112.

Gajda B, Grad I, Smoraą Z. 2009. *Effect of plant protein on development and quality of cultured in vitro porcine zygotes*. Reprod Fertil Dev 21(1):157–157.

Gandhi AP, Lane M, Gardner DK, Krisher RL. 2001. *Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media*. Mol Reprod Dev 58:269–275.

Gandolfi F. 1994. *Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst*. Theriogenology 41:95–100.

García-Vázquez FA, Matás C. 2010. *Cultivo embrionario in vitro en la especie porcina (I).* Cria y salud porcina 33:40-45.

Gardner DK, Lane M. 1993. *Amino acids and ammonium regulate mouse embryo development in culture.* Biol Reprod 48(2):377-385.

Gardner, DK. 1994. *Culture of mammalian embryos in the absence of serum and somatic cells*. Cell Biol Int 18:1163-1179.

Gardner DK, Lane M, Spitzer A, Barr PA. 1994. *Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development.* Biol Reprod 50(2):390-400.

Gardner DK, Lane M. 1996. *Alleviation of the "2-cell block" and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters.* Hum Reprod 11(12):2703-2712.

Gardner DK. 1998. *Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture.* Theriogenology 49(1):83-102.

Gardner, DK. 1999. *Development of serum-free culture system for the ruminant embryo and subsequent assessment of embryo viability*. J Reprod Fertil Suppl 54:461-475.

Gardner DK, Rodriegez-Martinez H, Lane M. 1999. *Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer*. Hum Reprod 14(10):2575-2580.

Gardner DK, Pool TB, Lane M. 2000. *Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability*. Semin Reprod Med 18(2):205-218.

Gardner DK, Larman MG, Thouas GA. 2010. *Sex-related physiology of the preimplantation embryo*. Mol Hum Reprod 16(8):539-547.

Gardón JC, Agüera S, Castejón F. 2004. Sexing in vitro produced bovine embryos, at different stages of development, using rat H-Y antiserum. 62(1-2):35-43.

Garner DL, Evans KM, Seidel GE. 2013. *Sex-sorting sperm using flow cytometry/cell sorting*. Methods Mol Biol 927:279-295.

Geshi M, Yonai M, Sakaguchi M, Nagai T. 1999. *Improvement of in vitro co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with beta-mercaptoethanol*. Theriogenology 51:551–558.

Gil MA, Abeydeera LR, Day BN, Vazquez JM, Roca J, Martinez EA. 2003. *Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs*. Theriogenology 60:767-776.

Gil MA, Cuello C, Parrilla I, Vazquez JM, Roca J, Martinez EA. 2010. *Advances in swine in vitro embryo production technologies*. Reprod Domest Anim 45:40–48.

Good AH, Cooper DK, Malcolm AJ, Ippolito RM, Koren E, Neethling FA, Ye Y, Zuhdi N, Lamontagne LR. 1992. *Identification of carbohydrate structures that bind human antiporcine antibodies: implications for discordant xenografting in humans*. Transplant Proc 24(2):559–562.

Goodall H, Roberts AM. 1976. *Differences in motility of human X- and Y-bearing spermatozoa*. J Reprod Fertil 48:433-436.

Gopichandran N, Leese HJ. 2006. *The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos*. Reproduction 131(2):269-277.

Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW. 2008. *Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone*. Biol Reprod 78: 812-815.

Greenlee AR, Krisher RL, Plotka ED. 1998. *Rapid sexing of murine preimplantation embryos using a nested, multiplex polymerase chain reaction (PCR)*. Mol Reprod Dev. 49:261-267.

Grossfeld R, Klinc P, Sieg B, Rath D. 2005. *Production of piglets with sexed semen employing a non-surgical insemination technique*. Theriogenology 63(8):2269-2277.

Grupen CG, Nagashima H, Nottle MB. 1995. *Cysteamine enhances in vitro development of porcine oocytes matured and fertilized in vitro.* Biol Reprod 53:173-178.

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. 1990. *A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes*. Nature 346(6281):245-250.

Gubbay J, Vivian N, Economou A, Jackson D, Goodfellow P, Lovell-Badge R. 1992. *Inverted repeat structure of the Sry locus in mice*. Proc Natl Acad Sci USA 89(17):7953-7957.

Gutiérrez-Adán A, Pérez G, Granados J, Garde JJ, Pérez-Guzmán M, Pintado B, 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.

Gutiérrez-Adán A, Oter M, Martínez-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 blatocyst stage*. Mol Reprod Dev 55(2):146-151.

Gutiérrez-Adán 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.

Gutiérrez-Adán A, Perez-Crespo M, Fernandez-Gonzalez R, Ramirez MA, Moreira P, Pintado B, Lonergan P, Rizos D. 2006. *Developmental consequences of sexual*

dimorphism during pre-implantation embryonic development. Reprod Domest Anim 41(2):54–62.

Hake L, O'Connor C. 2008. *Genetic mechanisms of sex determination*. Nature Education 1(1):25.

Hamashima N. 1982. *Effect of hyaluronic acid on the pre-implantation development of mouse embryo in vitro*. Dev Growth Differ 24:353–357.

Han YM, Wang WH, Abeydeera LR, Petersen AL, Kim JH, Murphy C, Day BN, Prather RS. 1999. *Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos*. Biol Reprod 61:1340-1346.

Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. 1989. *Biopsy of human preimplantation embryos and sexing by DNA amplification*. Lancet 1(8634):347-349.

Hansen D, Moller H, Olsen J. 1999. *Severe periconceptional life events and the sex ratio in offspring: Follow up study based on five national registers*. Bmj 319:548–549.

Hardarson T, Hanson C, Sjogren A, Lundin K. 2001. *Human embryos with unevenly blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation.* Hum Reprod 16: 313-318.

Hardy K, Hooper MA, Handyside AH, Tutherford AJ, Winston RM, Leese HJ. 1989. *Non invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos*. Human Reproduction 4:188–191.

Hardy K, Spanos S, Becker D, Iannelli P, Winston RML, Stark J. 2001. *From cell death to embryo arrest: mathematical models of human preimplantation development*. Proc Natl Acad Sci USA 98:1655-1660.

Harper JC, Coonen E, Ramaekers FC, Delhanty JD, Handyside AH, Winston RM, Hopman AH. 1994. *Identification of the sex of human preimplantation embryos in two hours using an improved spreading method and fluorescent in-situ hybridization (FISH) using directly labelled probes*. Hum Reprod 9(4):721-724.

Harvey AJ, Kind KL, Thompson JG. 2002. *REDOX regulation of early embryo development*. Reproduction 123:479–486.

Hashem A, Hossein MS, Woo JY, Kim S, Kim JH, Lee SH, Koo OJ, Park SM, Lee EG, Kang SK, Lee BC. 2006. *Effect of potassium simplex optimization medium and NCSU23 supplemented with beta-mercaptoethanol and amino acids of in vitro fertilized porcine embryos.* J Reprod Dev 52(5):591-599.

Hawkins JR, Taylor A, Goodfellow PN, Migeon CJ, Smith KD, Berkovitz GD. 1992. *Evidence for increased prevalence of SRY mutations in XY females with complete rather than partial gonadal dysgenesis*. Am J Hum Genet 51(5):979-984.

Hazeleger W, Kemp B. 1999. *State of the art in embryo transfer*. Theriogenology 51:81–90.

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.

Ho Y, Wiggesworth K, Eppig JJ, Schultz RM. 1995. *Preimplantation development of mouse embryo in KSOM: augmentation by amino acids and analysis of gene expression*. Mol Reprod Dev 41:232-238.

Hohenboken W. 1999. *Applications of sexed semen in cattle production*. Theriogenology 52:1421.

Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. 1998. *Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex*. Theriogenology 50(8):1285-1299.

Holte J, Berglund L, Milton K, Garello C, Gennarelli G, Revelli A, Bergh T. 2007. *Construction of an evidence-based integrated morphology cleavage embryo score for implantation potential of embryos scored and transferred on day 2 after oocyte retrieval*. Hum Reprod 22:548-557.

Hossein MS, Hashem MA, Jeong YW, Lee MS, Kim S, Kim JH, Koo OJ, Park SM, Lee EG, Park SW, Kang SK, Lee BC, Hwang WS. 2007. *Temporal effects of alpha-tocopherol and L-ascorbic acid on in vitro fertilized porcine embryo development.* Anim Reprod Sci 100(1-2):107-117.

Hosseini SM, Forouzanfar M, Hajian M, Asgari V, Abedi P, Hosseini L, Ostadhosseini S, Moulavi F, Safahani Langrroodi M, Sadeghi H, Bahramian H, Eghbalsaied Sh, Mohammad HNE. 2009. *Antioxidant supplementation of culture medium during embryo development and/or after vitrificaation-warming:which is the most important?* J Assist Reprod Genet 26(6):335-364.

Hu J, Cheng D, Gao X, Bao J, Ma X, Wang H. 2012. *Vitamin C enhances the in vitro development of porcine pre-implantation embryos by reducing oxidative stress.* Reprod Domest Anim 47(6):873-879.

Huang Y, Tang X, Xie W, Zhou Y, Li D, Zhou Y, Zhu J, Yuan T, Lai L, Pang D, Ouyang H. 2011. *Vitamin C enhances in vitro and in vivo development of porcine somatic cell nuclear transfer emrbyos.* Biochem Biophyd Res Commun 411(2):397-401.

Huck UW, Labov JB, Lisk RD. 1986. *Food restricting young hamsters (Mesocricetus auratus) affects sex ratio and growth of subsequent offspring*. Biol Reprod 35:592–598.

Huck UW, Pratt NC, Labov JB, Lisk RD. 1988. *Effects of age and parity on litter size and offspring sex ratio in golden hamsters (Mesocricetus auratus)*. J Reprod Fertil 83:209–214.

Huxtable RJ. 1992. *Physiological actions of taurine*. Physiol Rev 72(1):101-163.

Hyttel P, Viuff D, Fair T, Laurincik J, Thomsen PD, Callesen H, Vos PL, Hendriksen PJ, Dielman SJ, Shellander K, Besenfelder U, Greve T. 2001. *Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos*. Reproduction 122:21–30.

Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, Prather RS. 2004. *In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres*. Theriogenology 61:1125-1135.

Im GS, Yang BS, Lai L, Liu Z, Hao Y, Prather RS. 2005. *Fragmentation and development of preimplantation porcine embryos derived by parthenogenetic activation and nuclear transfer*. Mol Reprod Dev 71:159-165.

Iritani A, Sato E, Nishikawa Y. 1974. *Secretion rates and chemical composition of oviduct and uterine fluids in sows.* J Anim Sci 39(3):582-588.

Iritani A, Niwa K, Imai H. 1978. *Sperm penetration in vitro of pig follicular oocytes matured in culture.* J Reprod Fertil 54:379–383.

Ishikawa H, Rattigan A, Fundele R, Burgoyne PS. 2003. *Effects of sex chromosome dosage on placental size in mice*. Biol Reprod 69:483–488.

Isom SC, Li RF, Whitworth KM, Prather RS. 2012. *Timing of first embryonic cleavage is a positive indicator of the in vitro developmental potential of porcine embryos derived from in vitro fertilization, somatic cell nuclear transfer and parthenogenesis.* Mol Reprod Dev 79(3):197-207.

Iwasaki T, Kimura E, Totsukawa K. 1999. *Studies on a chemically defined medium for un vitro cultura of in vitro matured and fertilized porcine oocytes.* Theriogenology 51(4):709-720.

Iwata H, Kimura K, Hashimoto S, Ohta M, Tominaga T, Minami N. 2002. *Role of G6PD activity on sex ratio and developmental competence under oxidative stress.* J Reprod Dev 48:447–453.

Iwata H, Shiono H, Kon Y, Matsubara K, Kimura K, Kuwayama T, Monji Y. 2008. *Effects of modification of in vitro fertilization techniques on the sex ratio of the resultant bovine embryos*. Anim Reprod Sci 105:234-244.

Jang HY, Kong HS, Choi KD, Jeon J, Yang BK, Lee CK, Lee HK. 2005. *Effects of melatonin on gene expression of IVM/IVF porcine embryos*. Asian-Aust J Anim Sci 18:17-21.

Jansen RP, Burton GJ. 2004. *Mitochondrial dysfunction in reproduction*. *Mitochondrion* 4:577-600.

Jarrell VL, Day BN, Prather RS. 1991. *The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, Sus scrofa: Quantitative and qualitative aspects of protein synthesis*. Biol Reprod 44:62-68.

Jeon Y, Jeong SH, Biswas D, Jung EM, Jeung EB, Lee ES, Hyun SH. 2011. *Cleavage pattern and survivin expression in porcine embryos by somatic cell nuclear transfer.* Theriogenology 76(7):1187-1196.

Jiménez A, Madrid-Bury N, Fernández R, Pérez-Garnelo S, Moreira P, Pintado B, de la Fuente J, Gutiérrez-Adán A. 2003. *Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos*. Mol Reprod Dev 65:180–187.

Johnson LA, Clarke RN. 1988. *Flow sorting of X and Y chromosome-bearing mammalian sperm: activation and pronuclear development of sorted bull, boar, and ram sperm microinjected into hamster oocytes*. Gamete Res 21(4):335-343.

Johnson LA. 1997. *Advances in gender preselection in swine*. J Reprod Fertil Suppl 52:255-266.

Johnson LA, Rath D, Vazquez JM, Maxwell WM, Dobrinsky JR. 2005. *Preselection of sex of offspring in swine for production: current status of the process and its application*. Theriogenology 63(2):615-624.

Johnson MH, Nasr-Esfahani MH. 1994. *Radical solutions and cultural problems: could free oxygen radicals be responsable for the impaired developement of preimplantation mammalian embryos in vitro?* Bioessays 16(1):31-38.

Ka HH, Sawai K, Wang WH, Im KS, Niwa K, 1997. *Amino acids in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated in vitro*. Biol Reprod 57:1478-1483.

Kaminski MA, Ford SP, Youngs CR, Conley AJ. 1996. *Lack of effect of sex on pig embryonic development in vivo*. J Reprod Fertil 106:107–110.

Kano K, Miyano T, Kato S. 1998. *Effects of glycosaminoglycans on the development of in vitro-matured and -fertilized porcine oocytes to the blastocyst stage in vitro*. Biol Reprod 58:1226–1232.

Karja NWK, Medvedev S, Onishi A, Fuchimoto DI, Iwamoto M, Otoi T, Nagai T. 2004. *Effect of replacement of pyruvate/lactate in culture medium with glucose on preimplantation development of porcine embryos in vitro*. J Reprod Dev. 50:587-592.

Karja NW, Kikuchi K, Fahrudin M, Ozawa M, Somfai T, Ohnuma K, Noguchi J, Kaneko H, Nagai T. 2006. *Development to the blastocyst stage, the oxidative state, and the quality of early developmental stage of porcine embryos cultured in alteration of*

glucose concentrations in vitro under different oxygen tensions. Reprod Biol Endocrinol 4:54.

Karkanaki A, Praras N, Katsikis I, Kita M, Panidis D. 2007. *Is the Y chromosome all that is required for sex determination?* Hippokratia 11(3):120-123.

Kątska-Książkiewicz L. 2006. *Pig embryo production by in vitro maturation and fertilization of ovarian oocytes. A review.* J Anim Feed Sci 15:525–542.

Katz-Jaffe MG, Schoolcraft WB, Gardner DK. 2006. *Analysis of protein expression (secretome) by human and mouse preimplantation embryos*. Fertil Steril 86:678-685.

Kawarasaki T, Matsumoto K, Chikyu M, Itagaki Y, Horiuchi A. 2000. *Sexing of porcine embryo by in situ hybridization using chromosome Y- and 1-specific DNA probes*. Theriogenology 15;53(7):1501-1509.

Kawarsky SJ. 1999. *Effects of elevated temperature on bovine oocytes and embryos cultured in vitro*. Doctoral thesis, University of Guelph, Guelph, Ontario Canada.

Kidson, A. 2004. *In vitro embryo development in the pig: impact of oocyte maturation milieu on blastocyt morphology and viability*. Doctoral thesis, Utrecht University, Faculty of Veterinary Medicin. ISBN 90-393-3730-6

Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H. 1999. *Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured in vitro*. Biol Reprod 60: 336-340.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. 2002. *Successful piglet production after transfer of blastocysts produced by a modified in vitro system*. Biol Reprod 66:1033-1041.

Kim HS, Lee GS, Hyun SH, Lee SH, Nam DH, Jeong YW, Kim S, Kang SK, Lee BC, Hwang WS. 2004. *Improved in vitro development of porcine embryos with different energy substrates and serum*. Theriogenology 61:1381–1393.

Kim HS, Lee GS, Hyun SH, Nam DH, Lee SH, Jeong YW, Kim S, Kim JH, Kang SK, Lee BC, Hwang WS. 2005a. *Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos*. Theriogenology 63:1167–1180.

Kim HS, Lee GS, Kim HS, Jeong YW, Kim JH, Kang SK, Lee BC, Hwang WS. 2005b. *Embryotropic effect of insulin-like growth factor (IGF)-I and its receptor on development of porcine preimplantation embryos produced by in vitro fertilization and somatic cell nuclear transfer.* Mol Reprod Dev 72(1):88-97.

Kim JH, Niwa K, Lim JM, Okuda K. 1993. *Effects of phosphate, energy substrates, and amino acids on development of in vitro-matured, in vitro-fertilized bovine oocytes in a chemically defined, protein-free culture medium.* Biol Reprod 48(6):1320-1325.

Kim JY, Kim SB, Park MC, Park H, Park YS, Park HD, Lee JH, Kim JM. 2007. *Addition of macromolecules to PZM-3 culture medium on the development and hatching of in vitro porcine embryos.* Asian-Aust J Anim Sci 20(12):1820-1826.

Kimura K, Spate LD, Green MP, Roberts RM. 2005. *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 72:201–207.

Kimura K, Iwata H, Thompson JG. 2008. *The effect of glucosamine concentration on the development and sex ratio of bovine embryos*. Anim Reprod Sci 103(3-4):228-238.

King WA, Picard L, Bousquet D, Goff AK. 1992. *Sex-dependent loss of bisected bovine morulae after culture and freezing*. J Reprod Fertil 96:453-459.

Kircher RL, Lane M, Bavister BD. 1999. *Developmental competence and metabolism of bovine embryos cultured in semi-defined culture media*. Biol Reprod 60:1345-1352.

Kitagawaa Y, Suzukib K, Yonedaa A, Watanabea T. 2004. *Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos*. Theriogenology 62:1186–1197.

Klymiuk N, Aigner B, Brem G, Wolf E. 2010. *Genetic modification of pigs as organ donors for xenotransplantation.* Mol Reprod Dev 77(3):209-221.

Knox RV. 2011. *The current value of frozen-thawed boar semen for commercial companies*. Reprod Domest Anim 46(2):4-6.

Knox RV. 2014. Impact of swine reproductive technologies on pig and global food production. Adv Exp Med Biol 752:131-160.

Kobayashi J, Sekimoto A, Uchida H, Wada T, Sasaki K, Sasada H, Umezu M, Sato E. 1998. *Rapid detection of male-specific DNA sequence in bovine embryos using fluorescence in situ hybridization*. Mol Reprod Dev 51(4):390-394.

Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, Nakanishi T, Ikawa M, Hamada H, Abe K, Okabe M. 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.

Kochhar HP, Peippo J, King WA. 2001. *Sex related embryo development*. Theriogenology 55:3-14.

Kochhar HS, Kochhar KP, Basrur PK, King WA. 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.

Kolber-Simonds D, Lai L, Watt SR, Denaro M, Arn S, Augenstein M, Betthauser J, Carter DB, Greenstein JL, Hao Y, Im GS, Liu Z, Mell GD, Murphy CN, Park KW, Rieke A, Ryan DJ, Sachs DH, Forsberg EJ, Prather RS, Hawley RJ. 2004. *a1,3-galactosyltransferase null pigs via nuclear transfer with fibroblasts bearing loss of heterozygosity mutations*. Proc Natl Acad Sci USA 19:7335–7340.

Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. 1991. *Male development of chromosomally female mice transgenic for Sry*. Nature 351(6322):117-121.

Korhonen K, Julkunen H, Kananen K, Bredbacka P, Tiirikka T, Raty M, Vartia K, Kaimio I, Kontinen A, Halmekyto M, Vikki J, Peippo J, Lindeberg H. 2012. *The effect of ascorbic acid during biopsy and cryopreservation on viability of bovine embryos produced in vivo*. Theriogenology 77(1):201-205.

Kovtun IV, Welch G, Guthrie HD, Hafner KL, McMurray CT. 2004. *CAG repeat lengths in X- and Y-bearing sperm indicate that gender bias during transmission of Huntington's disease gene is determined in the embryo*. J Biol Chem 279:9389-9391.

Krietsch WK, Fundele R, Kuntz GW, Fehlau M, Burki K, Illmensee K. 1982. *The expression of X-linked phosphoglycerate kinase in the early mouse embryo*. Differentiation 23:141–144.

Kunieda T, Xian M, Kobayashi E, Imamichi T, Moriwaki K, Toyoda Y. 1992. *Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction*. Biol Reprod 46(4):692-697.

Kurebayashi S, Miyake M, Katayama M, Miyano T, Kato S. 1995. *Improvement of developmental abilty to the blastocyst stage by addition of hyaluronic acid to*

chemically defined medium in diploid porcine eggs matured in-vitro and subsequently electro-activated. J Mamm Ova Res 12:119–125.

Lane M, Gardner DK. 1992. *Effect of incubation volumen and embryo density on the development and viability of mouse embryos in vitro.* Hum Reprod 7(4):558-562.

Lane M, Gardner DK. 1996. *Selection of viable mouse blastocysts prior to transfer using metabolic criterion*. Hum Reprod 11:1975–1978.

Lane M, Gardner DK. 1997. *Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compactation of mouse zygotes in vitro.* Assist Reprod Genet 14:398-403.

Lane M, Gardner DK. 1998. *Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts.* Hum Reprod 13(4):991-997.

Lane M, Gardner DK. 2000. *Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo*. Biol Reprod 62:16-22.

Lane M, Hooper K, Gardner DK. 2001. Effect of essential amino acids on mouse embryo viability and ammonium production. J Assist Genet 18:519-525.

Lane M, Maybach JM, Gardner DK. 2002. *Addition of ascorbate during cryopreservation stimulates subsequent embryo development.* Hum Reprod 17(10):2686-2693.

Lane M, Gardner DK, Hasler MJ, Hasler JF. 2003. *Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture*. Theriogenology 60: 407-419.

Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. 2003. *Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan.* Mol Reprod Dev 64(1):70-78.

Lane M, Gardner DK. 2007. *Embryo culture medium: which is the best?* Best Prac Res Clin Obstet Gynaecol 21(1):83-100.

Lansac J, Thepot F, Mayaux MJ, Czyglick F,Wack T, Selva J, Jalbert P. 1997. *Pregnancy outcome after artificial insemination or IVF with frozen semen donor: a collaborative study of the French CECOS Federation on 21,597 pregnancies*. European J Obstet Gynecol Reprod Biol 74:223–228.

Larrabee PB, Johnson KL, Pestova E, Lucas M, Wilber K, LeShane ES, Tantravahi U, Cowan JM, Bianchi DW. 2004. *Microarray analysis of cell-free fetal DNA in amniotic fluid: a prenatal molecular karyotype*. Am J Hum Genet 75(3):485-491.

Larson M, Kimura K, Kubisch H, Roberts R. 2001. *Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocysts and in the expression of the signaling molecule IFN-Tau*. Proc Natl Acad Sci USA 98:9677–9682.

Latham KE, Patel B, Bautista FD, Hawes SM. 2000. *Effects of X chromosome number and parental origin on X-linked gene expression in preimplantation mouse embryos*. Biol Reprod 63:64–73.

Laurent TC, Fraser JRE. 1992. *Hyaluronan*. FASEB J 6:2397–2404.

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. 2002. *Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome.* Biol Reprod 67(3):767-775.

Le Bas-Bernardet S, Anegon I, Blancho G. 2008. *Progress and prospects: Genetic engineering in xenotransplantation*. Gene Ther 15:1247–1256.

Lechniak D, Kaczmarek D, Stanisławski D, Adamowicz T. 2002. *The ploidy of in vitro matured bovine oocytes is related to the diameter*. Theriogenology 57:1303-1308.

Lechniack D, Pers-Kamczyc E, Pawlak P. 2008. *Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos.* Reprod Biol 8(1):23-42.

Lee CN, Ax RL. 1984. *Concentration and composition of glycosaminoglycans in the female bovine reproductive tract*. J Dairy Sci 67:2006–2009.

Lee MS, Kang SK, Lee BC, Hwang WS. 2005. *The beneficial effects of insulin and metformin on in vitro developmental potential of porcine oocytes and embryos.* Biol Reprod 73:1264-1268.

Lee YS, Ock SA, Cho SK, Jeon BG, Kang TY, Balasubramanian S, Choe SY, Rho GJ. 2007. *Effect of donor cell type and passage on preimplantation development and apoptosis in porcine cloned embryos*. Asian-Aust J Anim Sci 20:711-717.

Leese HJ, Barton AM. 1984. *Pyruvate and glucose uptake by mouse ova and preimplantation embryos.* J Reprod Fertil 72(1):9-13.

Leese HJ. 1988. *The formation and function of oviduct fluid.* J Reprod Fertil 82(2):843-856.

Leese HJ, Baumann CG, Brison DR, Mc Evoy TG, Sturmey RG. 2008. *Metabolism of the viable mammalian embryo: Quietness revisited*. Mol Hum Reprod 14:667–672.

Leoni G, Ledda S, Bogliolo L, Naitana S. 2000. *Novel approach to cell sampling from preimplantation ovine embryos and its potential use in embryonic genome analysis.* J Reprod Fertil 119:309-314.

Lequarré AS, Vigneron C, Ribaucour F, Holm P, Donny I, Dalbies-Tran R, Callesen H, Mermillod P. 2005. *Influence of antral follicle size on oocyte characteristics and embryo development in the bovine*. Theriogenology 63:841–859.

Li RF, Wen LH, Wang SY, Bou S. 2006. *Development, freezability and amino acid consumption of bovine embryos cultured in synthetic oviductal fluid (SOF) medium containing amino acids at oviductal or uterine-fluid concentrations*. Theriogenology 66(2):404–414.

Li R, Lai L, Wax D, Hao Y, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Turk JR, Kang JX, Witt WT, Dai Y, Prather RS. 2006. *Cloned transgenic swine via in vitro production and cryopreservation.* Biol Reprod 75(2):226-230.

Li R, Whitworth K, Lai L, Wax D, Spate L, Murphy CN, Rieke A, Isom C, Hao Y, Zhong Z, Katayama M, Schatten H, Prather RS. 2007. *Concentration and composition of free amino acids and osmolalities of porcine oviductal and uterine fluid and their effects on development of porcine IVF embryos.* Mol Reprod Dev 74(9):1228-1235.

Li J, Li R, Liu Y, Villemoes K, Purup S, Callesen H. 2013. *Developmental kinetics of pig embryos by parthenogenetic activation or by handmade cloning.* Reprod Domest Anim 48(5):866-873.

Lloyd RE, Romar R, Matás C, Gutiérrez-Adán A, Holt WV, Coy P. 2009. *Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced in vitro*. Reproduction 137(4):679-687.

Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. 1996. *Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro*. Biol Reprod 54(6):1420-1429.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. 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, Gutierrez-Adan A, Pintado B, Fair T, Ward F, Fuente JD, Boland M. 2000. *Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro*. Mol Reprod Dev 57:146-152.

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP. 2003. *Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns.* Reprod Dom Anim 38:259-267.

Lonergan P, Pedersen HG, Rizos D, Greve T, Thomsen PD, Fair T, Evans A, Boland MP. 2004. *Effect of post-fertilization culture environment on the incidence of chromosome aberrations in bovine blastocysts.* Biol Reprod 71(4):1096-1100.

Lopes RF, Forell F, Oliveira AT, Rodrigues JL. 2001. *Splitting and biopsy for bovine embryo sexing under field conditions.* Theriogenology 56(9):1383-1392.

Lopes AS, Wrenzycki C, Ramsing NB, Herrmann D, Niemann H, Lovendahl P, Greve T, Callesen H. 2007. *Respiration rates correlate with mRNA expression of G6PD and GLUT1 genes in individual bovine in vitro-produced blastocysts.* Theriogenology 68:223-236.

Loutradis D, Drakakis P, Kallianidis K, Sofikitis N, Kallipolitis G, Milingos S, Makris N, Michalas S. 2000. *Biological factors in culture media affecting in vitro fertilization, preimplantation embryo development, and implantation.* Ann N Y Acad Sci 900:325-335.

MacCauley TC, Mozza MR, Didion BA, Mao J, Wu G, Coppola G, Coppola GF, Derrardino DD, Day BN. 2003. *Chromosomal abnormalities in day-6 in vitro produced pig embryos*. Theriogenology 34:1569-1580.

Machaty Z, Day BN, Prather RS. 1998. *Development of early porcine embryos in vitro and in vivo*. Biol Reprod 59:451-455.

Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N. 2004. *Reactivation of the paternal X chromosome in early mouse embryos*. Science 303:666–669.

Makarevich AV, Chrenek P, Flak P. 2006. *The influence of microinjection of foreign gene into the pronucleus of fertilized egg on the preimplantation development, cell number and diameter of rabbit embryos*. Asian-Aust J Anim Sci 19:171-175.

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

Mara L, Pilichi S, Sanna A, Accardo C, Chessa B, Chessa F, Dattena M, Bomboi G, Cappai P. 2004. *Sexing of in vitro produced ovine embryos by duplex PCR*. Mol Reprod Dev 69:35-42.

Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. 2001. *Meiotic and developmental competence of prepubertal and adult swine oocytes.* Theriogenology 56:17-29.

Marquant-Leguienne B, Humblot P. 1998. *Practical measures to improve in vitro blastocyst production in the bovine*. Theriogenology 49(1):3-11.

Martinez EA, Caamaño JN, Gil MA, Rieke A, McCauley TC, Cantley TC, Vazquez JM, Roca J, Vazquez JL, Didjon BA, Murphy CN, Prather RS, Day BN. 2004. *Successful nonsurgical deep uterine embryo transfer in pigs*. Theriogenology 61:137–146.

Martinez EA, Vazquez JM, Roca J, Cuello C, Gil MA, Parrilla I, Vazquez JL. 2005. *An update on reproductive technologies with potential short-term application in pig production.* Reprod Domest Anim 40(4):300-309.

Matás C, Coy P, Romar R, Marco M, Gadea J, Ruiz S. 2003. *Effect of sperm preparation method on in vitro fertilization in pigs*. Reproduction 125(1):133-141.

Mattioli M, Bacci ML, Galeati G, Seren E. 1989. *Developmental competence of pig oocytes matured and fertilized in vitro*. Theriogenology 31:1201-1207.

Maurer, HR. 1992. *Towards serum-free, chemically defined media for mammalian cell culture*. In: Freshney RI Ed. Animal Cell Culture: A Practical Approach. University Press, Oxford, UK. pp 15-46.

McGraw RA, Jacobson RJ, Akamatsu M. 1988. *A male-specific repeated DNA sequence in the domestic pig*. Nucleic Acids Res 16:10389.

McKiernan SH, Bavister BD. 1992. *Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos*. In vitro Cell Dev Biol 28:154-156.

Medvedev S, Onishi A, Fuchimoto DI, Iwamoto M, Nagai T. 2004. *Advanced in vitro production of pig blastocysts obtained through determining the time for glucose supplementation*. J Reprod Dev 50:71-76.

Meikle DB, Drickamer LC. 1986. *Food availability and secondary sex ratio variation in wild and laboratory house mice (Mus musculus)*. J Reprod Fertil 78:587–591.

Memorial University of Newfoundland. 2014. Available on-line via: <http://www.mun.ca/biology/scarr/4241_Devo_MammalSex.html> [Accessed: January 8, 2014]

Men H, Agca Y, Critser ES, Critser JK. 2005. *Beneficial effects of serum supplementation during in vitro production of porcine embryos on their ability to survive cryopreservation by open pulled straw vitrification*. Theriogenology 64:1340–1349.

Ménézo YJ, Chouteau J, Torello J, Girard A, Veiga A. 1999. *Birth weight and sex ratio after transfer at the blastocyst stage in humans*. Fertil Steril 72:221–224.

Ménézo YJ. 2006. *Paternal and maternal factors in preimplantation embryogenesis: interaction with the biochemical environment*. Reprod Biomed Online 12:616–621.

Ménézo YJ, Lichtblau I, Elder K. 2013. *New insights into human pre-implantation metabolism in vivo and in vitro.* J Assist Reprod Genet 30:293-303.

Miller JG, Schultz GA. 1997. *Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract.* Biol Reprod 36(1):125-129.

Minami N, Suzuki T, Tsukamoto S. 2007. *Zygotic gene activation and maternal factors in mammals*. J Reprod Dev 53:707-715.

Misener M, Pollard JW, Metzger K. 1991. *In vitro culture of porcine embryos in CZB medium*. Theriogenology 35:244 (Abstract).

Mittwoch U. 1985. *Erroneous theories of sex determination*. J Med Genet 22:164-170.

Mittwoch U. 2004. *The elusive action of sex-determining genes: mitochondria to the rescue?* J Theor Biol 228:359-365.

Mittwoch U. 2005. *Sex determination in mythology and history*. Arquivos brasileiros de endocrinologia e metabologia 49:7-13.

Miyano T, Hino-oka RE, Kano K, Miyake M, Kusunokir H, Katoi S. 1994. *Effects of hyaluronic acid on the development of 1- and 2-cell porcine embryos to the blastocyst stage in vitro*. Theriogenology 41: 1299–1305.

Miyoshi K, Umezu M, Sato E. 1999. *Effect of hyaluronic acid on the development of porcine 1-cell embryos produced by a conventional or new in vitro maturation/fertilization system*. Theriogenology 51:777–784.

Mizuno J, Ostrovidov S, Nakamura H, Akaishi K, Inui H, Sakai Y, Fujii T, Anzai K, Watanabe A. 2007. *Human ART on chip: development of microfluidic device for IVF and IVC*. Hum Reprod 22:432.

Monk M, Handyside AH. 1988. *Sexing of preimplantation mouse embryos by measurement of X-linked gene dosage in a single blastomere*. J Reprod Fertil 82(1):365-368.

Moreira F, Paula-Lopes FF, Hansen PJ, Badinga L, Thatcher WW. 2002. *Effects of growth hormone and insulin-like growth factor-I on development of in vitro derived bovine embryos*. Theriogenology 57:895–907.

Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard M-A. 2006. *The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels*. Mol Reprod Dev 73:1367–1379.

Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH. 2006. *Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance afterslow freezing or vitrification.* Theriogenology 65(8):1551-1562.

Munné S, Weier HU, Stein J, Grifo J, Cohen J. 1993. *A fast and efficient method for simultaneous X and Y in situ hybridization of human blastomeres*. J Assist Reprod Genet 10(1):82-90.

Nagai T, Funahashi H, Yoshioka K, Kikuchi K. 2006. *Up date of in vitro production of porcine embryos.* Front in Biosci 11:2565–2573.

Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Nottle MB. 1995. *Cryopreservation of porcine embryos*. Nature 374:416.

Nagashima H, Hiruma K, Saito H, Tomii R, Ueno S, Nakayama N, Matsunari H, Kurome M. 2007. *Production of live piglets following cryopreservation of embryos derived from in vitro-matured oocytes*. Biol Reprod 76:900–905.

Nagyova E, Vanderhyden BC, Prochazka R. 2000. *Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes.* Biol Reprod 63:1149–1156.

Nakazawa Y, Misawa H, Fujino Y, Tajima S, Misumi K, Ueda J, Nakamura Y, Shibata T, Hirayama Y, Kikuchi K. 2008. *Effect of volume of non-surgical embryo transfer medium on ability of porcine embryos to survive to term.* J Reprod Dev 54:30–34.

Naqvi AN. 2007. *Application of molecular genetic technologies in livestock production: potentials for developing countries.* Adv Biol Res 1(3-4):72-84.

Nedambale TL, Dinnyés A, Yang X, Tian XC. 2004. *Bovine blastocyst development in vitro: timing, sex, and viability following vitrification*. Biol Reprod 71:1671–1676.

Ng E, Claman P, Leveille MC, Tanphaichitr N, Compitak K, Suwajanakorn S, Wells G. 1995. *Sex ratio of babies in unchanged after transfer of fast- versus slow-cleaving embryos*. J Assoc Reprod Genet 12:566–568.

Nichol R, Hunter HF, Gardner DK, Leese HJ, Cooke GM. 1992. *Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period*. J Reprod Fertil 96:669–707.

Niemann H, Rath D. 2001. *Progress in reproductive biotechnology in swine.* Theriogenology 56(8):1291-1304.

Niemann H, Rath D, Wrenzycki C. 2003. *Advances in biotechnology: new tools in future pig production for agriculture and biomedicine*. Reprod Domest Anim 38:82–89.

O'Neil C. 1997. *Evidence of the requirement of autocrine growth factors for development of mouse preimplantation embryo in vitro*. Biol Reprod 56:229–237.

Ober C, Loisel DA, Gilad Y. 2008. *Sex-specific genetic architecture of human disease*. Nat Rev Genet 9(12):911-922.

Ock SA, Kim JG, Shi LY, Jin HF, Mohana Kumar B, Choe SY, Rho GJ. 2005. *Comparison of development and quality of porcine embryos cultured in different oxygen concentrations*. Reprod Fertil Dev 17:223 (abstract).

Okada K, Krylov V, Kren R, Fulka J. 2006. *Development of pig embryos after electro-activation and in vitro fertilization in PZM-3 or PZM supplemented with fetal bovine serum*. J Reprod Dev52:91-98.

Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E. 2004. *Epigenetic dynamics of imprinted X inactivation during early mouse development.* Science 303:644–649.

Okere C, Nelson L. 2002. *Novel reproductive techniques in swine production.* Asian-Aust J Anim Sci 15(2):445-452.

Oliver G, Novak S, Patterson JL, Pasternak JA, Paradis F, Norrby M, Oxtoby K, Dyck MK, Dixon WT, Foxcroft GR. 2011. *Restricted feed intake in lacting primiparous sows. II. Effects on subsequent litter sex ratio and embryonic gene expression*. Reprod Fertil Dev 23(7):899-911.

Olson SE, Seidel GE. 2000. *Culture of in vitro-produced bovine embryos with Vitamin-E improves development in vitro and transfer to recipients*. Biol Reprod 62:248-252.

Orsi NM, Leese HJ. 2004. *Amino acid metabolism of preimplantation bovine embryos cultured with bovine serum albumin or polyvinyl alcohol.* Theriogenology 61(1-2):561-572.

Oxenreider SL, Day BN. 1965. *Transport and cleavage of ova in swine.* J Anim Sci 24:413-417.

Palasz AT, Alkemade S, Mapletoft RJ. 1993. *The use of sodium hyaluronate in freezing media for bovine and murine embryos*. Cryobiology 30:172–178.

Palasz AT, Rodriguez-Martinez H, Beltran-Breña P, Pérez-Garnelo S, Martínez MF, Gutiérrez-Adán A, De la Fuente J. 2006. *Effects of hyaluronan, BSA, and serum on bovine embryo in vitro development, ultrastructure, and gene expression patterms*. Mol Reprod Dev 73:1503–1511.

Palma GA, Clement-Segewald A, Berg U, Brem G. 1992. *Role of the embryo number in the developmentof in vitro produced bovine embryos*. Theriogenology 37:271 (Abstract).

Pantaleon M, Kaye PL. 1996. *IGF-I and insulin regulate glucose transport in mouse blastocysts via IGF-I receptor*. Mol Reprod Dev 44:71–76.

Pantaleon M, Tan HY, Kafer GR, Kaye PL. 2010. *Toxic effects of hyperglycemia are mediated by the hexosamine signaling pathway and O-linked glycosylation in early mouse embryos*. Biol Reprod 82:751–758.

Paria BC, Dey SK. 1990. *Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors*. Proc Natl Acad Sci USA 87(12):4756-4760.

Park JH, Lee JH, Choi KM, Joung SY, Kim JY, Chung GM, Jin DI, Im KS. 2001. *Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere*. Theriogenology 55(9):1843-1853.

Parrilla I, Vazquez JM, Gil MA, Caballero I, Almiñana C, Roca J, Martinez EA. 2004. *Influence of storage time on functional capacity of flow cytometrically sex-sorted boar spermatozoa*. Theriogenology 64(1):86-98.

Peippo J, Bredbacka P. 1995. *Sex-related growth rate differences in mouse preimplantation embryos in vivo and in vitro*. Mol Reprod Dev 40(1):56-61.

Peippo J, Huhtinen M, Kotilainen T. 1995. *Sex diagnosis of equine preimplantation embryos using the polymerase chain reaction*. Theriogenology 44(5):619-627.

Penfold LM, Holt C, Holt WV, Welch GR, Cran DG, Johnson LA. 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.

Pérez-Crespo M, Ramírez MA, Fernández-González R, Rizos D, Lonergan P, Pintado B, Gutiérrez-Adán A. 2005. *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(4):502-510.

Perner S, Bruderlein S, Hasel C, Waibel I, Holdenried A, Ciloglu N, Chopurian H, Nielsen KV, Plesch A, Hogel J, Moller P. 2003. *Quantifying telomere lengths of human individual chromosome arms by centromere-calibrated fluorescence in situ hybridization and digital imaging*. Am J Pathol 163:1751–1756.

Pers-Kamczyc E, Pawlak P, Rubes J, Lechniak D. 2012. *Early cleaved bovine embryos show reduced incidence of chromosomal aberrations and higher developmental potential on day 4.5 post-insemination*. Reprod Domest Anim 47:889-906.

Peters JK, Milliken G, Davis L. 2001. *Development of porcine embryos in vitro: effects of culture medium and donor age.* J Anim Sci 79:1578-1583.

Petters RM, Johnson BH, Reed ML, Archibong AE. 1990. *Glucose, glutamine and inorganic phosphate in early development of the pig embryo in vitro*. J Reprod Fertil 89:269–275.

Petters RM, Wells KD. 1993. *Culture of pig embryos*. J Reprod Fertil Suppl 48:61–73.

Pfeifer TL, Chegini N. 1994. *Immunohistochemical localization of insulin-like growth factor (IGF-I), IGF-I receptor, and IGF binding proteins 1-4 in human fallopian tube at various reproductive stages.* Biol Reprod 50(2):281-289.

Phelps CJ, Koike C, Vaught TD, Boone J, Wells K, Chen S, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL. 2003. *Production of 1,3-galactosyltransferase-deficient pigs*. Science 299:411–414.

Pinto AB, Carayannopoulos MO, Hoehn A, Dowd L, Moley KH. 2002. *Glucose transporter 8 expression and translocation are critical for murine blastocyst survival*. Biol Reprod 66:1729–1733.

Polejaeva I, Chen S, Vaught T, Page R, Mullins J, Ball S, Dai Y, Boone J, Walker S, Avares DL, Colman A, Campbell KH. 2000. *Cloned pigs produced by nuclear transfer from adult somatic cells*. Nature 407:86–90.

Pomp D, Good BA, Geisert RD, Corbin CJ, Conley AJ. 1995. *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 73:1408–1415.

Prakash S, Patole MS, Ghumatkar SV, Nandode SK, Shinde BM, Shouche YS. 2000. *Mitochondrial 12S rRNA sequence analysis in wildlife forensics*. Curr Sci 78:1239-1241.

Prelle K, Stojkovic M, Boxhammer K, Motlik J, Ewald D, Arnold GJ, Wolf E. 2001. *Insulin-like growth factor I (IGF-I) and long R^3 IGF-I differently affect development and messenger ribonucleic acid abundance for IGF-binding proteins and type I IGF receptors in in vitro produced bovine embryos*. Endocrinology 142:1309–1316.

Probst S, Rath D. 2003. *Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes*. Theriogenology 59(3-4):961-973.

Pushpakumara PG, Robinson RS, Demmers KJ, Mann GE, Sinclair KD, Webb R, Wathes DC. 2002. *Expression of the insulin-like growth factor (IGF) system in the bovine oviduct at oestrus and during early pregnancy*. Reproduction 123(6):859-868.

Quintans CJ, Donaldson MJ, Blanco LA, Sergio Pasqualini R. 1998. *Deviation in sex ratio after selective transfer of the most developed cocultured blastocysts*. J Assist Reprod Genet 15:403–404.

Rath D, Johnson LA, Dobrinsky JR, Welch GR, Niemann H. 1997. *Production of piglets preselected for sex following in vitro fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry*. Theriogenology 47(4):795-800.

Rath D, Ruiz S, Sieg B. 2003. *Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen*. Vet Rec 152(13):400-401.

Rattanasuk S, Parnpai R, Ketudat-Cairns M. 2011. *Multiplex polymerase chain reaction used for bovine embryo sex determination.* J Reprod Dev 57(4):539-542.

Ray PF, Conaghan J, Winston RM, Handyside AH. 1995. *Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization*. J Reprod Fertil 104:165–171.

Reis A, Rooke JA, McCallum GJ, Staines ME, Ewen M, Lomax MA, McEvoy TG. 2003. *Consequence of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced in vitro*. Reprod Fertil Dev 15:275-284.

Rho SH, Hwang WS. 2002. *In vitro development of porcine parthenogenetic and cloned embryos: comparison of oocyte-activating techniques, various culture system and nuclear transfer methods*. Reprod Fertil Dev14:93-99.

Ried T, Landes G, Dackowski W, Klinger K, Ward DC. 1992. *Multicolor fluorescence in situ hybridization for the simultaneous detection of probe sets chromosomes 13, 18, 21, X and Y in uncultured amniotic fluid cells*. Hum Mol Genet 1:307-313.

Rieger D, Loskutoff NM, Betteridge KJ. 1992. *Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro*. Reprod Fertil Dev 4:547–557.

Riesenbeck A. 2011. *Review on international trade with boar semen*. Reprod Domest Anim 46(2):1-3.

Rivers JP, Crawford MA. 1974. *Maternal nutrition and the sex ratio at birth*. Nature 252:297–298.

Rizos D, Ward F, Duffy P, Boland MP, 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(2):234-248.

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. 2003. *Bovine embryo culture in the presence or absence of serum: implication for blastocyst development, cryotolerance, and messenger RNA expression*. Biol Reprod 68:236-243.

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

Rizos D, Clemente M, Bermejo-Alvarez P, de la Fuente J, Lonergan P, Gutiérrez-Adán A. 2008b. *Consequences of in vitro culture conditions on embryo development and quality.* Reprod Domest Anim 43(4):44-50.

Robl and Davis DL. 1981. *Effects of serum on swine morulae and blastocysts in vitro.* J Anim Sci 52(6):1450-1456.

Romek M, Gajda B, Krzytofowicz E, Kepczynski M, Smoraq Z. 2011. *Lipid content in pig blastocysts cultured in the presence or absence or protein and vitamin E or phenazine ethosulfate*. Folia Biol (Krakow) 59:45–52.

Rose RC, Bode AM. 1993. *Biology of free radical scavengers: an evaluation of ascorbate.* FASEB J 7(12):1135-1142.

Rosenfeld CS, Grimm KM, Livingston KA, Brokman AM, Lamberson WE, Roberts RM. 2003. *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 USA 100:4628–4632.

Rosenfeld CS, Roberts RM. 2004. *Maternal diet and other factors affecting offspring sex ratio: a review*. Biol Reprod 71(4):1063-1070.

Ross MT, Grafham DV, Coffey AJ, Scherer S, McLay K, Muzny D, Platzer M, Howell GR, Burrows C, Bird CP et al. 2005. *The DNA sequence of the human X chromosome*. Nature 434:325–337.

Rubessa M, Boccia L, Campanile G, Longobardi V, Albarella S, Tateo A, Zicarelli L, Gasparrini B. 2011. *Effect of energy source during culture on in vitro embryo development, resistance to cryopreservation and sex ratio*. Theriogenology 76:1347-1355.

Sathasivam K, Kageyama S, Chikuni K, Notarianni E. 1995. *Sex determination in the domestic pig by DNA amplification using the HMG-box sequence*. Anim Reprod Sci 38:321–326.

Schini SA, Bavister BD. 1988a. *Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose.* Biol Reprod 39(5):1183-1192.

Schmidt A, Einspanier R, Amselgruber W, Sinowatz F, Schams D. 1994. *Expression of insulin-like growth factor 1 (IGF-1) in the bovine oviduct during the oestrous cycle*. Exp Clin Endocrinol 102(5):364-369.

Schoenbeck RA, Peters MS, Rickords LF, Stumpf TT, Prather RS. 1992. *Characterization of deoxyribonucleic acid synthesis and the transition from maternal to embryonic control in the 4-cell porcine embryo*. Biol Reprod 47:1118-1125.

Seidel GE. 2003. *Economics of selecting for sex: the most important genetic trait*. Theriogenology. 59(2):585-598.

Sembon S, Suzuki S, Fuchimoto D, Iwamoto M, Kawarasaki T, Onishi A. 2008. *Sex identification of pigs using polymerase chain reaction amplification of the amelogenin gene*. Zygote 16(4):327-332.

Seshagiri PB, Bavister BD. 1989. *Glucose inhibits development of hamster 8-cell embryos in vitro.* Biol Reprod 40(3):599-606.

Seshagiri PB, Bavister BD. 1991. *Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the "crabtree effect".* Mol Reprod Dev 30(2):105-111.

Shea BF. 1999. *Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study*. Theriogenology 51:841–854.

Sigma-Aldrich Co. LLC. Copyright © 2014.
Available on-line via: <http://www.sigmaaldrich.com/spain.html>

[Accessed January 10, 2014]

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN. 1990. *A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif*. Nature 346(6281):240-244.

Smith LC, Alcivar AA. 1993. *Cytoplasmic inheritance and its effect on development and performance*. J Reprod Fertil 48:31-43.

Somfai T, Ozawa M, Noguchi J, Kaneko H, Karja NW, FAhrudin M, Nakai M, Maedomari N, Dinnyés A, Nagai T, Kikuchi K. 2008. *In vitro development of polyspermic porcine oocytes: Relationship between early fragmentation and excessive number of penetrating spermatozoa*. Anim Repod Sci 107:131-147.

Somfai T, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S, Konishi K, Imai K. 2010. *Relationship between the length of cell cycles, cleavage pattern and developmental competence in bovine embryos generated by in vitro fertilization or parthenogenesis*. J Reprod Dev 56:200-207.

Somfai T, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S, Akai T, Hattori H, Konishi K, Imai K. 2010. *Culture of bovine embryos in polyester mesh sections: the effect of pore size and oxygen tension on in vitro development*. Reprod Domest Anim 45(6):1104-1109.

Spicer LJ, Echternkamp SE. 1995. *The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals*. Domest Anim Endocrinol 12:223–245.

Spiteller G. 2001. *Peroxidation of linoleic acid and its relation to aging and agedependent diseases*. Mech Ageing Dev 122:617-657.

Squires EJ. 1999. *Genetics of boar taint: Implications for future use of intact males*. Available on-line via: National Swine Improvement Federation Conference and Annual Meeting proceedings. <http://www.nsif.com/Conferences/1999/squires.htm>
[Accessed: April 19, 2013]

Steele CC, Jefferys EH, Diplock AT. 1974. *The effect of vitamin E and synthetic antioxidants on the growth in vitro of explanted rat embryos*. J Reprod Fertil 38:115-123.

Steeves TE, Gardner DK. 1999. *Temporal and differential effects of amino acids on bovine embryo development in culture*. Biol Reprod 61(3):731-740.

Stojkovic M, Kölle S, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JG, Wenigerkind H, Reichenbach HD, Sinowatz F, Wolf E. 2002. Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embryos produced in vitro. Reproduction 124(1):141-153.

Stojkovic M, Krebs O, Kolle S, Prelle K, Assmann V, Zakhartchenko V, Sinowatz F, Wolf E. 2003. *Developmental regulation of hyaluronan-binding protein (RHAMM/IHABP) expression in early bovine embryos*. Biol Reprod 68:60–66.

Stokes PJ, Abeydeera LR, Leese HJ. 2005. *Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro*. Dev Biol 284(1):62-71.

Sturmey RG, Leese HJ. 2003. *Embryo metabolism in pig oocytes and early embryos.* Reproduction 126(2):197-204.

Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. 2010. *Amino acid metabolism of bovine blastocysts: a biomarker of sex viability*. Mol Reprod Dev 77(3):285-296.

Suchanek E, Simunic V, Juretic D, Grizelj V. 1994. *Follicular fluid contents of hyaluronic acid, follicle-stimulating hormone and steroids relative to the success of in vitro fertilization of human oocytes*. Fertil Steril 62:347–352.

Summers MC, Biggers JD. 2003. *Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues.* Hum Reprod Update 9(6):557-582.

Sutton-McDowall ML, Gilchrist RB, Thompson JG. 2010. *The pivotal role of glucose metabolism in the determining oocyte developmental competence*. Reproduction 139(4):685-695.

Swain JE, Bormann CL, Clark SG, Walters EM, Wheeler MB, Krisher RL. 2002. *Use of energy substrates by various stage preimplantation pig embryos produced in vivo and in vitro*. Reproduction 123:253-260.

Swain JE, Smith GD. 2011. *Advances in embryo culture platforms: novel approaches to improve preimplantation embryo development through modifications of the microenvironment.* Hum Reprod Update 17(4):541-557.

Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A. 1993. *Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos.* Biol Reprod 49(2):228-232.

Takahashi M, Nagai T, Okamura N, Takahashi H, Okano A. 2002. *Promoting effect of beta-mercaptoethanol on in vitro development under oxidative stress and cystine uptake of bovine embryos.* Biol Reprod 66(3):562-567.

Tappel AI. 1980. *Vitaminum E and selenium from in vivo lipid peroxidation*. Ann NY Acad Sci 355:98-108.

Tarin JJ, Bernabeu R, Baviera A, Bonada M, Cano A. 1995. *Sex selection may be inadvertently performed in in-vitro fertilization-embryo transfer programmes*. Hum Reprod 10:2992–2998.

Taylor DM, Handyside AH, Ray PF, Dibb NJ, Winston RM, Ao A. 2001. *Quantitative measurement of transcript levels throughout human preimplantation development: Analysis of hypoxanthine phosphoribosyl transferase*. Mol Hum Reprod 7:147–154.

Terlouw SL, Dobrinsky JR. 2010. *Pig embryo transfer: a glimpse into the future*. Pig Progress 26(3):14-16.
Available on-line via Minitube International. <http://www.minitube.de/DE_eng/Media/Library/Published-Papers>
[Accessed: February 11, 2012]

Thompson JG, Simpson AC, Pugh PA, Pugh RW, Tervit HR. 1991. *Glucose utilization in sheep embryos derived in vivo and in vitro*. Reprod Fertil Dev 3:571–576.

Thomson JG, Peterson AJ. 2000. *Bovine embryo culture in vitro: new developments and post-transfer consequences.* Hum Reprod Suppl 15:59-67.

Tiemann U, Viergutz T, Jonas L, Wollenhaupt K, Pöhland R, Kanitz W. 2001. *Fluorometric detection of platelet activating factor receptor in cultured oviductal epithelial and stromal cells and endometrial stromal cells from bovine at different stages of the oestrous cycle and early pregnancy*. Domest Anim Endocrinol 20(3):149-164.

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.

Toyokawa K, Harayama H, Miyake M. 2005. *Exogenous hyaluronic acid enhances porcine parthenogenetic embryo development in vitro possibly mediated by CD44*. Theriogenology 64:378–392.

Trivers RL, Willard DE. 1973. *Natural selection of parental ability to vary the sex ratio of offspring*. Science 179(4068):90-92.

Ufer C, Wang CC. 2011. *The roles of glutathione peroxidise during embryo development*. Front Mol Neurosci 4:12.

Ulloa Ulloa CM, Yoshizawa M, Komoriya E, Mitsui A, Nagai T, Kikuchi K. 2008. *The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in in vitro produced porcine embryos*. J Reprod Dev 54:22-29.

Vajta G, Rienzi L, Cobo A, Yovich J. 2010. *Embryo culture: can we perform better tan nature?* Reprod Biomed Online 20(4):453-469.

Van Soom A, Vandaele L, Goossens K, de Kruif A, Peelman L. 2007. *Gamete origin in relation to early embryo development*. Theriogenology 68(1):131-137.

Van Soom A, Ysebaert MT, de Kruif A. 1997. *Relationship between timing of development, morula, morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos.* Mol Reprod Dev 47(1):47-56.

Van Thuan N, Harayama H, Miyake M. 2002. *Characteristics of preimplantational development of porcine parthenogenetic diploids relative to the existence of amino acids in vitro*. Biol Reprod 67(6):1688-1698.

van Vliet RA, Verrinder Gibbins AM, Walton JS. 1989. *Livestock embryo sexing: a review of current methods, with emphasis on Y-specific DNA probes*. Theriogenology 32:421–438.

Van Winkle LJ. 2001. *Amino acid transport regulation and early embryo development*. Biol Reprod 64(1):1-12.

Vandaele L, Mateusen B, Maes D, de Kruif A,Van Soom A. 2006. *Is apoptosis in bovine in vitro produced embryos related to early developmental kinetics and in vivo bull fertility*. Theriogenology 65:1691-1703.

Vandaele L, Mateusen B, Maes DG, de Kruif A, Van Soom A. 2007. *Temporal detection of caspase-3 and -7 in bovine in vitro produced embryos of different developmental capacity*. Reproduction 133:709-718.

Vazquez JM, Martinez EA, Parrilla I, Roca J, Gil MA, Vazquez JL. 2003. *Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa*. Theriogenology 59(7):1605-1614.

Veerhuis R, Hendriksen PJ, Hengst AM, Kruijt L, Tieman M, Booman P. 1994. *The production of anti-H-Y monoclonal antibodies: their potential use in a sex test for bovine embryos*. Vet Immunol Immunopathol 42(3-4):317-330.

Vinsky MD, Novak S, Dixon WT, Dyck MK, Foxcroft GR. 2006. *Nutritional restriction in lactating primiparous sows selectively affects female embryo survival and overall litter development*. Reprod Fertil Dev 18(3):347-355.

Wang WH, Abeydeera LR, Cantley TC, Day BN. 1997. *Effect of oocyte maturation media on development of pig embryos produced by in vitro fertilization*. J Reprod Fert 111:101-108.

Wang H, Rodriguez-Osorio N, Feugang JM, Jung SY, Garrison K, Wolgemuth C, Greer L, CrenShaw M, Memili E. 2009. *Effects of culture media and inhibitors on biology of porcine early embryonic development in vitro.* Livestock Science 121:102-107.

Wang Q, Zhang X, Zhang HY, Zhang J, Chen GQ, Zhao DH, Ma HP, Liao WJ. 2010. *Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene*. Meat Sci 85:265–269.

Ward F, Rizos D, Corridan D, Quinn K, Boland M, Lonergan P. 2001. *Paternal influence on the time of the first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo.* Mol Reprod Dev 60(1):47-55.

Weimar CH, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. 2013. *Reprint of: in-vitro model systems for the study of human embryo-endometrium interactions.* Reprod Biomed Online 27(6):673-688.

Williams TJ. 1986. *A technique for sexing mouse embryos by a visual colorimetric assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase*. Theriogenology 25(5):733-739.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. 1999. *Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA.* Mol Reprod Dev 53(1):8-18.

Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. 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.

Wyman A, Pinto AB, Sheridan R, Moley KH. 2008. *One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring*. Endocrinology 149:466–469.

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.

Xu JS, Chan ST, Lee WW, Lee KF, Yeung WS. 2004. *Differential growth, cell proliferation, and apoptosis of mouse embryo in various culture media and incoculture*. Mol Reprod Dev 68(1):72-80.

Yadav BR, King WA, Betteridge KJ. 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.

Yang YG, Sykes M. 2007. *Xenotransplantation: Current status and a perspective on the future*. Nat Rev Immunol 7:519–531.

Yeste M, Briz M, Pinart E, Sancho S, García-Gil N, Badia E, Bassols J, Pruneda A, Bussalleu E, Casas I, Bonet S. 2008. *Hyaluronic acid delays boar sperm capacitation after 3 days of storage at 15ºC*. Anim Reprod Sci 109: 236-250.

Yeste M. 2013a. *Boar spermatozoa within the oviductal environment (I): sperm reservoir*. In: Bonet S, Casas I, Holt WV, Yeste M Eds. Boar Reproduction: Fundamentals and New Biotechnological Trends. Ed. Springer: Berlin, Germany. pp 257-346. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8.

Yeste M. 2013b. *Boar spermatozoa within the oviductal environment (III): fertilization*. In: Bonet S, Casas I, Holt WV, Yeste M Eds. Boar Reproduction: Fundamentals and New Biotechnological Trends. Ed. Springer: Berlin, Germany. pp 407-467. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8.

Yoshida M, Mizoguchi Y, Ishigaki K, Kojima T, Nagai T. 1993. *Birth of piglets derived from in vitro fertilization of pig oocytes matured in vitro.* Theriogenology 39:1303-1311.

Yoshioka K, Suzuki C, Tanaka A, Anas IMK, Iwamura S. 2002. *Birth of piglets derived from porcine zygotes cultured in a chemically defined medium*. Biol Reprod 66:112-119.

Yoshioka K, Suzuki C, Itoh S, Kikuchi K, Iwamura S, Rodriguez-Martinez H. 2003. *Production of piglets derived from in vitro-produced blastocysts fertilized and*

cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during in vitro fertilization. Biol Reprod 69:2092-2099.

Yoshioka K, Suzuki C, Onishi A. 2008. *Defined system for in vitro production of porcine embryos using a single basic medium.* J Reprod Dev 54(3):208-213.

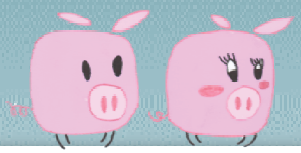
Yoshioka K. 2011. *Development and application of a chemically defined medium for the in vitro production of porcine embryos.* J Reprod Dev 57(1):9-16.

Yoshioka K, Noguchi M, Suzuki C. 2012. *Production of piglets from in vitro-produced embryos following non-surgical transfer.* Anim Reprod Sci. 131(1-2):23-29.

Youngs CR, McGinnis LK. 1990. *In vitro culture of porcine embryos in Whitten's medium containing varying levels of glucose and bovine serum albumin*. Biol Reprod 42:58 (Abstract).

Yuh HS, Yu DH, Shin MJ, Kim HJ, Bae KB, Lee DS, Lee HC, Chang WK, Park SB, Lee SG, Park HD, Ha JH, Hyun BH, Ryoo ZY. 2010. *The effects of various antioxidants on the development of parthenogenetic porcine embryos*. In Vitro Cell Dev Biol Anim 46:148–154.

Zhang W, Yi K, Yan H, Zhou X. 2012. *Advances on In vitro production and cryopreservation of porcine embryos.* Anim Reprod Sci 132:115-122.



Universitat de Girona

Institut de Tecnologia Agroalimentària (INTEA)

Parc Científic i Tecnològic Universitat de Girona