UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Physiological Approach for the Optimization of the *In Vitro* Embryo Production System in Swine

Aproximación Fisiológica para la Optimización del Sistema de Producción de Embriones *In Vitro* en la Especie Porcina

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2019

STUDY FUNDING

This study was funded by Spanish Ministry of Economy and Competitiveness (MINECO) and European Regional Development Fund (FEDER). Grants AGL2012-40180-C03-01 and AGL2015-66341-R.

Soledad García Martínez benefited from aid Erasmus + Practices (Erasmus +, European Comission) to a doctoral stay at the Epigenetic programme at Babraham Institute (Cambridge, UK). From September 1st 2015 to December 31th 2015.

Soledad García Martínez benefited from an EPICONCEPT COST action Short Term Scientific Missions (STSM). (Reference code: COST-STSM-ECOST-STSM-FA1201-150716-079344) as visiting student at the Epigenetic programme at Babraham Institute (Cambridge, UK). From 15/07/2016 to 15/09/2016. From July 15th 2016 to September 15th 2016.

Soledad García Martínez benefited from aid Ayudas para estancias en el extranjero de jóvenes investigadores y estudiantes de doctorado en las líneas de actuación de Campus Mare Nostrum (Reference code: R-421/2017) to a doctoral stay at Facoltà di Bioscienze e Tecnologie Agro-Alimentari ed Ambientali, Università di Teramo (UNITE) (Teramo, Italy). From July 1st 2017 to September 30 th 2017.

ACKNOWLEDGEMENTS

The time is now right for the presentation of our Doctoral Thesis. Yeah "our" you read it right because this academic work could not have been achieved without the active cooperation of a wide group of people and institutions. It is therefore, my great pleasure and honour to use this space to dedicate a few words to each one of them.

First, I must specially thank my supervisors, Prof. Pilar Coy, Prof. Raquel Romar and Prof. Nicola Bernabò, for accepting me to do this PhD Thesis under their leadership. This work is much theirs as mine. Thanks for designing, planning and guiding this work with great dedication and professionalism, as well as, providing me with the materials and infrastructures to perform it. Prof. Pilar, I came to you being an inexperienced college without any real vocation, but you helped me to discover the fascinating world of science. I must admit that your professional expertise and accomplishments were very inspiring, but what truly led me, in a way, to the completion of this PhD thesis on your side was your modesty and positive ways of thinking. Thank you so much for showing me my vocational path. Prof. Raquel, thank you for your willingness to help, for being a clear, precise and coherent person and for taking care of the slightest detail of this work. Thank you so much for having your company on this amazing journey. Prof. Nicola, I would like to thank you for welcoming me very kindly in your laboratory during my PhD stay in Italy. I learned a lot from your experience and knowledge. Thank you for designing and helping me within the temperature experiments.

I would like to extend my sincere appreciations to each of the members that are part of the Department of Physiology of the Faculty of Veterinary, University of Murcia, Spain. A multicultural, multidisciplinary and welcoming research group from whom I have learned a lot over the years. To the professors Pilar, Raquel, Carmen, Joaquín, Salvador and Francisco Alberto. Thank you all for improving my research training and your always-accurate suggestions. To the collaborating Departments, such as the Department of Anatomy and Comparative Pathology of the Faculty of Veterinary, University of Murcia, Spain. Especially professors Octavio Albors and Rafael Latorre, key players within the collection and analysis of oxygen and temperature data measured in the reproductive tract of female pigs. At this point, I would also like to include the

Scientific Director of the Laparoscopy Unit of the Minimally Invasive Surgery Center Jesús Usón, Cáceres, Spain, Francisco Miguel Sánchez Margallo, as well as the Dr. Miguel Ángel Sánchez Hurtado.

I would like to thank the generous and disinterested assistance of the Doctorate and Master students with whom I have shared long hours of laboratory and friendship. To the first ones, Irene Jon and Cristina, in particular Cristina, for being "my test tube mom". To those who arrived later, Silvia, Analuce, Evelynne, Jordana and Daniel. To the last ones, Sergio, Alexia and Chiara. I would also like to thank all of you whom were passed through since I started in the lab, you all know who you are, and I am truly grateful for all you have done. I would specially like to mention the important role of the technician team (Juan Antonio, Soledad and Evelynne) in the achievement of the PhD thesis' goals.

I would like to thank the members of the Faculty of Veterinary Medicine, Università degli Study di Teramo, Italy by the warm reception and invaluable help, particularly to Marina, Juliana, Gulia and Luca. At this point, I would also like to consider Dr. Gavin Kelsey and Dr. Elena Ivanova from Babraham Institute, Cambridge, United Kingdom, and thank them for helping me to take my first steps in epigenetics.

Lastly, I would like to express my deepest gratitude to my family. To my parents (Isidro and Sole), for providing me with a safe space to develop and grow. Thank you for providing me with education and teaching me the values that govern my life today. To my older and always protective sister (Nuria), thank you for accompanying me and helping me to remove the obstacles that I have been facing in my path, you are and always will be my wholehearted support. Dani, you have recently arrived to my life but you have become a very important part of it. Thank you for your patience, support and for bringing me so much happiness.

INDEX

FIGURE INDEX

TABLE INDEX

ABSTRACT

The *in vitro* embryo production (IVEP), which includes obtaining gametes followed by *in vitro* fertilization (IVF) and embryo culture (EC) for subsequent transfer of the embryos to the uterus or their use for research purposes, is one of the most important reproductive biotechnologies in recent times. Its importance bases on the possibilities offered by its use in clinical, biomedical and agriculture fields. In the clinic, this technology has become a tool widely used to treat infertility problems. In biomedicine, the production of zygotes or embryos from animals with genetic, anatomical and physiological characteristics similar to humans, such as pig, is relevant to the study and treatment of diseases, as well as to the development of biotechnologies such as xenotransplantation, transgenesis or cloning. Finally, in agriculture, it allows the genetic improvement of livestock breeds for human consumption and creates a path to sustainability. However, all these advantages are limited by the low efficiency of the IVEP systems; and despite the numerous studies and the progress made to date, the *in vitro* embryo formation rates in mammals are below 50%. Another great disadvantage of this biotechnology is the low quality of the embryos produced *in vitro* compared with those obtained naturally within the female reproductive tract, being essential to improve the quality of the *in vitro* embryos for obtaining healthy individuals, free of diseases during postnatal development.

Among the most used assisted reproductive technologies (ART) in pigs, artificial insemination (AI) and superovulation are highlighted. However, embryo transfer (ET) does not present the same acceptance, due to the problems associated with the use of *in vitro* produced embryos, among other issues. The main drawbacks in porcine IVEP are the high rates of polyspermy, entrance of two or more sperm to the oocyte, during IVF and the poor development of the *in vitro* grown embryos. Those issues place the efficiency of porcine IVEP below 30 %.

Thanks to advances in the epigenetic field, the impact of an artificial environment on the embryonic genotype and phenotype can be studied. This, together with the data provided by comparative studies between *in vitro* and *in vivo* embryos, point to the

importance of studying the differences between the artificial and the natural environment and how mimicking this last one during artificial conditions, may bring about an improvement in IVEP. For approximating the procedures carried out in the pig IVEP to what happens *in vivo*, we will have to take into account the factors influencing the environment of the reproductive tract of the female pig. The function of some of them has already been studied; however, a deeper analysis of other factors is still needed.

The pre-ovulatory oviductal fluid where gametes are found during the fertilization is responsible for feeding those cells during that process. In addition, according to numerous studies, this fluid is able to modify the surface of the gametes taking part in the interaction process between the spermatozoa and the oocyte. One of the main roles attributed to this fluid is its ability to modify the oocytes' zona pellucida (ZP) in different species, including the pig, making it more resistant ("hardest") to proteolytic digestion and therefore, reducing the polyspermy rates during IVF. Within this fluid, the Oviductal specific glycoprotein (OVGP1 or oviductin), together with heparin, have been identified as responsible for this hardening. However, other proteins such as ezrin, HSP-70-1A and HSP-90α have been described in that fluid. This means that these proteins might contribute to the hardening effect as well; however, their involvement in that process has not been yet studied. On the other hand, the uterine fluid, in which the embryos are immersed once they reach the uterus, should have a relevant role on embryonic development. Despite this fact, very few studies using uterine fluid during ART have been reported.

Another main factor that affects fertilization and embryo development is the physical-chemical environment that surrounds gametes and embryos during different reproductive events. This ambient is characterised by the different conditions of temperature, pH and gases composition within the reproductive organs and these ones may vary between species. Then, it is necessary to know the values of these conditions (reference values) within the reproductive organs of the different species in order to transfer them to the ART. Nowadays, the oxygen and temperature reference values used during ART are far from the *in vivo* value, according to the few existing studies. In fact, at present, there are no oxygen reference values in the reproductive tract of the female

pig, nor are there any temperature reference values in the uterus of this species, although a temperature gradient in the oviduct and ovary has been previously recorded. Today, the existence of leading-edge technology and new devices offered by the market would allow us to obtain reference values of oxygen and temperature in those organs. For example, in the case of oxygen, the concentration used during IVF and CE in this species is the atmospheric one (20%), despite the studies indicating a negative effect of its use on embryonic development and pregnancy rates in different species of mammals. In the case of temperature, the thermal gradients recorded in the pig ovary and oviduct indicate an important role of temperature within different reproductive events given in those organs. However, *in vitro* maturation, IVF and EC take place at the same temperature (38.5 °C), and the impact of minimal variations of temperature during porcine IVEP is not taken into account.

The main hypothesis of the present study suggest that an approximation of ART environment to the physiological ambient found in the female reproductive tract could help to overcome some of the ART-derived problems. This last idea in line with the objectives of this PhD Thesis tries to approach the IVF and CE conditions to those recorded in the sow's reproductive tract for the improvement of the *in vitro* production of porcine embryos. To this end, four experiments have been carried out. The first two consisted of the modifications in the culture media used during IVF and CE by adding either specific proteins or natural fluids present in the oviduct and uterus of sows. The last two experiments consisted of obtaining *in vivo*, inside the reproductive organs of the female pig, the reference values for physical-chemical parameters such as oxygen and temperature, and transfer them to *in vitro* conditions.

In the first experiment, the effect of ezrin, HSP-70-1A and HSP-90α, together with heparin, on the hardening of porcine oocytes' zona pellucida (ZP), and different parameters evaluated after IVF, were studied. To do this, porcine oocytes matured *in vitro* were incubated in a medium with heparin or with each of these proteins. These same groups were used during IVF since heparin or proteins were added to the IVF medium with the oocytes and spermatozoa. In the control groups, gametes were incubated in a medium without heparin nor proteins. The results showed that heparin and each of the

tested proteins hardened the ZP of the porcine oocytes, by increasing its resistance to enzymatic digestion. The combination of heparin with each of the proteins increased such hardening effect, except for HSP-90α, where the the ZP´s resistance was reduced. While promising, since the effect of heparin and each of the proteins added into the IVF medium drafted the polyspermy rates, IVF outcome was not improved because of the reduction in the penetration rates. For this reason, another experiment was designed, where reproductive fluids (oviductal and uterine), which contain all the proteins and multiple factors present under physiological conditions, were added to the culture media during sperm selection, IVF and EC.

In the second experiment, two sperm selection methods were compared. One of the widely used procedures in swine, the density gradient centrifugation, was compared with a new procedure, which made the sperm swam up in a tube containing culture media with or without porcine oviductal fluid (POF) in order to bring closer to *in vitro* conditions the changes suffered by spermatozoa in the sow reproductive tract. On the other hand, two IVEP systems were compared. A new system containing POF and porcine uterine fluid (PUF) within the culture media during IVF and EC was compared with a system where those biofluids were not added to the culture media. The results indicated that the use of swim-up as sperm selection procedure for the production of porcine embryos reached a 40% yield, indicating an improvement with regard to the best results obtained in pigs up to date (30-35%). Furthermore, that value increased in a 5% when the reproductive fluids were added to the different culture media, even surpassing the value of the yield given for the bovine species. Likewise, the addition of the fluids to the media improved the quality of the porcine *in vitro* embryos produced, which achieved a mean number of cells similar to porcine embryos collected *in vivo*.

In the third and fourth experiments, a series of measurements of oxygen and temperature were made in the reproductive tract of sows and gilts at different phases of the estrous cycle, using minimally invasive surgery techniques and new probes. The values obtained were then transferred to IVF and CE procedures, with the purpose of improving the production of porcine embryos *in vitro*.

The third experiment revealed the amount of oxygen within the reproductive organs of sow (7%), being higher in gilts treated with hormones (10%). Those reference values for oxygen in the reproductive organs of the female pig were retrieved for the first time. Two profiles for oxygen measurements were observed: a flat profile, where the oxygen variations were minimal with respect to the average, and a wavy profile, which showed small variations (± 2) with respect to the average. The wavy profile was associated with the uterus. The use of hypoxia conditions (7% oxygen) during IVF followed by EC improved porcine IVEP outcome and the quality of the porcine blastocysts obtained.

The fourth experiment was in disagreement with the existence of a temperature gradient between isthmus and ampulla, which was proposed more than three decades ago and remain unquestionable to this day. In contrast, it was demonstrated the existence of a temperature gradient between the oviduct (37.0 ºC) and uterus (38.7 ºC) for the first time. Transferring this gradient during IVF and CE improved the production of *in vitro* embryos in the porcine species through the reduction of the polyspermy rates. In addition, the use of a high temperature (39.5 ºC) during IVF exerted the opposite effect to the use of a low temperature (37.0 ºC), since its use increased the polyspermy rates, and a greater fragmentation of the embryos obtained at high temperature was observed. On the other hand, obtaining the transition temperature for capacitated boar sperm (37.0 ºC), as well as the incubation of these spermatozoa at different temperatures (37.0 ºC, 38.5 ºC and 39.5 ºC), indicated an important role of temperature in the remodelling of the lipids contained in the sperm membrane and its implication in IVF.

Overall, it is derived from the present work that the approximation of the pig ART, using porcine reproductive fluids and the oxygen and temperature conditions retrieved from the reproductive organs of the female pig, improves the output of this technology. Therefore, similar approaches could be carried out in other mammalian species of interest with the purpose of achieving the same goal.

RESUMEN

La producción de embriones *in vitro* (IVEP), que incluye la obtención de gametos seguida de la fecundación *in vitro* (IVF) y el cultivo de embriones (EC) para su posterior transferencia al útero o para fines de investigación, es una de las biotecnologías reproductivas más importantes de los últimos tiempos. Su importancia radica en las posibilidades que ofrece su uso en clínica, biomedicina y agricultura. En clínica, esta tecnología se ha convertido en una de las herramientas más recurrida para tratar los problemas de infertilidad. En biomedicina, la producción de zigotos o embriones procedentes de animales como el cerdo, con características genéticas, anatómicas y fisiológicas similares a humanos, es relevante para el estudio y tratamiento de enfermedades, así como para el avance en biotecnologías como xenotransplantes, transgénesis o clonación. Finalmente, en agricultura, permite la mejora genética de las razas ganaderas para consumo humano y ofrece alternativas de sistemas de producción más sostenibles y respetuosos con el medio ambiente. Sin embargo, todas estas ventajas se encuentran limitadas por el bajo rendimiento de los sistemas de IVEP; y es que a pesar de los numerosos estudios y progresos realizados hasta el momento, las tasas de formación de embriones *in vitro* en mamíferos se encuentran por debajo del 50 %. Otra de las grandes desventajas de esta biotecnología es la baja calidad de los embriones producidos *in vitro* comparada con aquella presente en los embriones que se desarrollan de manera natural en el tracto reproductor femenino. Producir embriones de calidad es fundamental para la obtención de individuos sanos, libres de enfermedades que puedan afectar al posterior desarrollo pos-natal.

Entre las técnicas de reproducción asistida (ART), en cerdos, destacan la inseminación artificial (AI) y la superovulación. Sin embargo, la transferencia embrionaria (ET) no presenta la misma aceptación debido a los problemas asociados a la utilización de embriones *in vitro*, entre otros. Los mayores inconvenientes encontrados durante la IVEP en la especie porcina son las altas tasas de polispermia, entrada de dos o más espermatozoides al ovocito, durante la IVF y el bajo desarrollo de los embriones

producidos en condiciones artificiales. Estos obstáculos hacen que las tasas de IVEP porcina se sitúen por debajo de 30 %.

Gracias al avance en el campo de la epigenética, se ha podido estudiar el impacto que tiene un ambiente artificial sobre el genotipo y fenotipo embrionario. Esto junto a los datos aportados por distintos estudios comparativos entre embriones desarrollados *in vitro* e *in vivo*, son indicadores de la importancia del estudio de las diferencias entre el ambiente artificial y el ambiente natural, y como un acercamiento de ese primer ambiente a este último podría suponer una mejora en la IVEP. Para aproximar los procedimientos llevados a cabo durante la IVEP porcina a aquello que ocurre *in vivo*, tendrían que tenerse en cuenta distintos elementos que se encuentran dentro del tracto reproductor de la cerda y que interaccionan, de alguna manera, con los gametos y embriones en los distintos eventos reproductivos. La función de alguno de ellos ya ha sido estudiada, sin embargo es necesario un análisis más profundo de otros de ellos.

El fluido oviductal pre-ovulatorio donde se encuentran embebidos los gametos durante la fecundación es el encargado de nutrir a estas células durante el proceso de fecundación. Además, según numerosos estudios, es capaz de modificar la superficie de los gametos e intervenir en el proceso de interacción ovocito – espermatozoide. Uno de los papeles más importantes atribuidos a este fluido es su capacidad de modificar la zona pelucida de ovocitos en diferentes especies, entre ellas el cerdo, haciéndola más resistente ("más dura") a la digestión proteolítica y por tanto, de reducir los niveles de polispermia durante la IVF. Dentro de ese fluido, la glicoproteína específica oviductal (OVGP1 u oviductina), junto con heparina, han sido identificadas como moléculas responsables de ese endurecimiento. No obstante, se han descrito otras proteínas como ezrin, HSP-70-1A y HSP-90α que se encuentran en gran proporción dentro del fluido pre-ovulatorio, y que por tanto, podrían contribuir a ese endurecimiento. Sin embargo, su implicación en ese proceso no ha sido estudiada. Por otro lado, el fluido uterino, en el cual se encuentran sumergidos los embriones una vez llegan al útero, debe tener un papel relevante durante el desarrollo embrionario. A pesar de ello, son muy pocos los estudios que lo tienen en cuenta durante las ART.

Otro de los principales factores que afecta a la fecundación y el desarrollo embrionario es el entorno físico-químico que rodea a los gametos y embriones durante los distintos eventos reproductivos. Este ambiente se caracteriza por unas determinadas condiciones de temperatura, pH y concentración de gases dentro de los órganos reproductores femeninos, las cuales pueden variar dependiendo de la especie. Es necesario conocer los valores de estas condiciones (valores de referencia) dentro de los órganos reproductores femeninos de las diferentes especies con el propósito de transferirlos a las ART. Las condiciones de oxígeno y temperatura que se utilizan *in vitro,* hoy en día, se encuentran alejadas de los valores de referencia registrados *in vivo,* de acuerdo a los pocos estudios existentes. De hecho, actualmente, no existen valores de referencia de oxígeno en el tracto reproductor de la cerda, ni valores de referencia de temperatura en el útero de esta especie, aunque sí se ha registrado un gradiente de temperatura en el oviducto y ovario. En la actualidad, la existencia de tecnología de vanguardia y los nuevos dispositivos que ofrece el mercado permitirían obtener valores de referencia de oxígeno y temperatura en esos órganos. Por ejemplo, en el caso del oxígeno, la concentración utilizada durante la IVF y EC en esta especie es la atmosférica (20 %), a pesar de los estudios que indican un efecto negativo del uso de esta concentración sobre el desarrollo embrionario y las tasas de embarazo en diferentes especies de mamíferos. En el caso de la temperatura, los gradientes térmicos descritos en el ovario y oviducto de cerdas indican que variaciones mínimas de temperatura tienen lugar dentro de esos órganos reproductivos, y por tanto, pueden tener un papel importante en distintos eventos que se dan en el interior de ellos. Sin embargo, la maduración *in vitro*, la IVF y el EC tiene lugar a la misma temperatura (38.5 ºC), y el impacto de esas oscilaciones de temperatura durante la IVEP porcina no se tiene en cuenta.

La hipótesis principal del presente estudio sugiere que la aproximación del entorno durante las ART al ambiente fisiológico que se encuentra en el tracto reproductor de la hembra podría ayudar a superar algunos de los problemas derivados de las ART. Esta última idea es el objetivo principal de esta Tesis Doctoral: la aproximación de las condiciones de IVF y EC a las registradas en el tracto reproductor de la cerda para la mejora de la producción *in vitro* de embriones en la especie porcina. Con este fin, se

realizaron cuatro experimentos. Los dos primeros consistieron en la modificación de los medios de cultivo utilizados durante la IVF y el EC mediante la adición de proteínas específicas o fluidos naturales presentes en el oviducto y el útero de la cerda. Los dos últimos experimentos consistieron en obtener *in vivo* los valores de referencia para parámetros físico-químicos como el oxígeno y temperatura del interior de los órganos reproductores de la cerda, y transferirlos a las condiciones *in vitro*.

En el primer experimento, se estudió el efecto de ezrin, HSP-70-1A y HSP-90α junto a heparina sobre el endurecimiento de la zona pelúcida de los ovocitos porcinos y distintos parámetros evaluados después de la IVF. Para ello, ovocitos porcinos madurados *in vitro* fueron incubados en un medio con heparina o cada una de estas proteínas. Estos mismos grupos fueron utilizados durante la IVF, ya que heparina o las proteínas fueron añadidas al medio de IVF donde se encontraban los ovocitos y espermatozoides. Los gametos fueron incubados en medio sin heparina y proteínas para los grupos control. Los resultados mostraron que heparina y cada una de las proteínas por sí mismas eran capaces de endurecer la zona pelúcida de los ovocitos porcinos, es decir, aumentar los tiempos de digestión para la zona pelúcida. La combinación de heparina con cada una de las proteínas aumentó esos tiempos, excepto para HSP-90α donde disminuyó. Aunque prometedores, ya que el efecto de heparina y cada una de las proteínas añadidas al medio de IVF redujeron las tasas de polispermia, los resultados también mostraron bajas tasas de penetración cuando estas moléculas fueron añadidas a los medios, de manera que no se consiguió la mejora de la técnica de IVF con este enfoque. Por lo que a continuación, se diseñó otro experimento, dónde se agregaron fluidos reproductivos (oviductal y uterino), que contienen todas las proteínas y múltiples factores presentes en condiciones fisiológicas, a los medios de cultivo durante la selección espermática, la IVF y el EC.

En el segundo experimento se compararon dos métodos de selección espermática. Uno de los métodos más utilizados para la especie porcina, centrifugación a través de un gradiente de densidad, se comparó con un nuevo procedimiento que hizo que los espermatozoides nadaran en un tubo que contenía un medio de cultivo con o sin fluido oviductal porcino (POF) con la intención de imitar los cambios sufridos por los espermatozoides en su ascenso por el tracto reproductor femenino. Por otro lado, se

compararon dos sistemas de producción de embriones. Un nuevo sistema de cultivo, donde se añadió de forma secuencial POF al medio de IVF, y POF y fluido uterino porcino (PUF) a los medios de EC, frente a un sistema de producción de embriones donde no se añadió estos fluidos a los medios. Los resultados indicaron que la utilización de swim-up como método de selección espermática para la producción de embriones porcinos alcanzó el 40 % de rendimiento, indicando una mejora con respecto a los mejores resultados previos registrados en cerdos (30 -35 %). Además, ese valor incrementó un 5 % cuando los fluidos reproductivos fueron añadidos a los distintos medios de cultivo, superando incluso el valor de rendimiento dado para la especie bovina. Igualmente, la adición de los fluidos a los medios mejoró la calidad de los embriones obtenidos, alcanzando un número medio de células similar al de embriones obtenidos *in vivo*.

En el tercer y cuarto experimento se realizaron una serie de mediciones de oxígeno y temperatura en el tracto reproductivo de cerdas adultas y cerdas pre-púberes, en distintas fases del ciclo estral, usando técnicas de cirugía y sondas mínimamente invasivas. A continuación, los valores obtenidos se transfirieron a las técnicas de IVF y EC, con el propósito de mejorar la producción de embriones porcinos *in vitro*.

El tercer experimento reveló por primera vez la cantidad de oxígeno dentro de los órganos reproductivos de la cerda adulta (7 %), siendo este valor mayor (10 %) para cerdas pre-púberes tratadas con hormonas. Se observaron dos perfiles para las medidas de oxígeno: perfil plano, donde la variación de oxígeno fue mínima respecto al promedio, y un perfil ondulado que presentó pequeñas variaciones (±2) respecto al promedio. El perfil ondulado fue asociado al útero. El uso de condiciones de hipoxia (7 % oxígeno) durante la IVF seguido del EC mejoró la producción de embriones porcinos y la calidad de los blastocistos obtenidos.

El cuarto experimento se mostró en desacuerdo con la existencia de un gradiente de temperatura entre istmo y ampolla, el cuál fue propuesto hace más de tres décadas y mantenido incuestionable hasta nuestros días. En cambio, demostró la existencia de un gradiente de temperatura entre oviducto (37.0 ºC) y útero (38.7 ºC) por primera vez. La transferencia de ese gradiente durante las técnicas de IVF y EC mejoró la producción de

embriones *in vitro* en la especie porcina a través de la reducción de los ratios de polispermia. El uso de una temperatura elevada (39.5 ºC) durante la IVF ejerció el efecto contrario al uso de una temperatura baja (37.0 ºC), ya que aumentó los ratios de polispermia y se pudo intuir una mayor fragmentación de los embriones producidos a esa temperatura. La obtención de la temperatura de transición para espermatozoides capacitados de verraco (37.0 ºC), así como la incubación de esos espermatozoides a distintas temperaturas (37.0 ºC, 38.5 ºC y 39.5 ºC) determinó un papel relevante de ésta en la remodelación de los lípidos de la membrana plasmática del espermatozoide y su implicación en la IVF.

En general, de este trabajo se puede extraer que la aproximación de las ART en el cerdo usando fluidos reproductivos y las condiciones de oxígeno y temperatura propias de la especie mejora el rendimiento de esta biotecnología. Por lo tanto, aproximaciones similares se podrían llevar a cabo en otras especies de mamíferos de interés para conseguir el mismo propósito.

1. INTRODUCTION, HYPOTHESIS AND OBJECTIVES

In vitro embryo production (IVEP) comprise a group of reproductive technologies that make it possible to obtain blastocysts on a large scale from manipulation of gametes and development of the zygotes and embryos in laboratory conditions. IVEP is primarily used as an infertility treatment in humans. In the case of animals, it is generally used to improve the number of offspring from selected individuals or for research purposes. The current possibility of using IVEP to insert, delete or replace genetic material to the genome of a species as swine that share similar genetic, anatomical, physiological and biochemical characteristics with humans, makes of this species the animal model par excellence of the 21st century for genetic engineering (Perleberg, Kind, & Schnieke, 2018). Nowadays, genetically modified pigs are used for the study of relevant biomedical applications such as xenotransplantation, gene therapy or production of therapeutic proteins as well as for the study of diseases as cystic fibrosis, Alzheimer, spinal muscular atrophy, diabetes or cardiovascular diseases (Hryhorowicz, Zeyland, Słomski, & Lipiński, 2017; Wheeler, 2003). Genetically modified pigs also have agricultural applications aimed, on the one hand, at obtaining low-fat pigs with greater resistance to diseases, being hence more suitable for human consumption; and, on the other hand, at reducing the concentration of potentially polluting by-products of livestock origin (Whyte & Prather, 2011). Therefore, these applications have recently made the porcine IVEP a scientific tool of particular interest to improve the biomedical field and the agrarian sector.

The *in vitro* production of porcine embryos implies the following laboratory steps:

1) Collection of the biological material. Porcine immature cumulus-oocyte complexes (COCs) are usually collected from ovarian follicles of slaughtered gilts or sows at the abattoir, and spermatozoa are obtained from ejaculates of proven fertility boars or epididymis of slaughtered boars.

2) Selection of good quality gametes. Not all the gametes collected in the first step are used to produce porcine *in vitro* embryos since the selection of high quality gametes to overcome the subsequent steps is crucial. For this reason, COCs are selected following morphological criteria, while spermatozoa are selected by using specific laboratory procedures that allow reducing the number of cells with low quality.

3) *In vitro* maturation (IVM) of COCs. This step takes place in suitable culture conditions that support both oocyte nuclear and cytoplasmic maturation. This process leads to the oocyte to complete the second meiosis and acquire the fertilization capability (Abeydeera, 2002). Porcine IVM has currently standardized protocols that achieve percentages of nuclearly mature oocytes above 90 % (Oberlender et al., 2013).

4) *In vitro* fertilization (IVF). This step consists of the co-incubation of mature COCs, usually previously denuded, with spermatozoa during a suitable period under controlled atmosphere. Porcine IVF has currently protocols that achieve an efficiency varying from 30 to 50 % in most IVF laboratories (Abeydeera, Wang, Cantley, Prather, & Day, 1998; Funahashi, Fujiwara, & Nagai, 2000; Gil et al., 2003; Gil et al., 2004; Romar, Funahashi, & Coy, 2016; Suzuki, Eriksson, Shimizu, Nagai, & Rodriguez-Martinez, 2000). This low efficiency range for porcine IVF is due mainly to the abnormal penetration of two or more spermatozoa into the oocyte, phenomenon known as "polyspermy", potentially lethal for the embryo and with an exceptionally high prevalence in swine.

5) The culture of the obtained zygotes up to the blastocyst stage, namely embryo culture (EC). The last step consists in the culture of the zygotes obtained in the previous step under controlled atmosphere for a maximum period of one week. The efficiency of porcine EC is poor, around 25 % of blastocyst formation, and the quality of the obtained blastocyst inferior, compared with their *in vivo* counterparts (Stokes, Abeydeera, & Leese, 2005). The low efficiency of the EC is attributed to the low IVF outcomes along with the sub-optimal *in vitro* culture (IVC) conditions.

The three last steps of the IVEP systems, mentioned above, IVM, IVF and EC are designed to supply the needs of each stage: COCs maturation, zygote, and embryo

development, respectively. While *in vivo*, these steps, together with the gametes selection, occur within the female reproductive tract, the *in vitro* environment is supported by different procedures, culture media and incubators, where nutrients and atmosphere are still far from those provided by the female tract and hence, still deficient. This is one of the reasons why manipulated gametes and *in vitro* embryos are especially vulnerable to the culture conditions during the previously mentioned steps. While porcine IVM shows a high efficiency, the environment provided by IVF and EC is far from being suitable for zygotes and embryo development indicating that they require further research.

Therefore, this work was focussed on developing a more reliable *in vitro* production system for porcine embryos based on the **HYPOTHESIS that the use of *in vitro* conditions closer to those found within the reproductive tract of the female pig would be able to improve the IVEP outcomes**. In view of the need for studies in this regard, special attention was paid in this work to the IVC environment during IVF and EC.

To contrast the hypothesis of this work, the selection of few of the main parameters forming the natural microenvironment in swine to study their impact on porcine IVEP was made. This natural environment embraces a complex mix of oviductal and uterine secretions, as well as physic-chemical parameters such as pH, temperature or gas composition that are physiologically modified depending on the animal reproductive status. These natural fluctuations make this microenvironment hard to emulate during *in vitro* conditions. Some of the main approaches that should be overcome in IVEP of swine and that have been covered in this work are described below.

The first part of this work has been based on the study of Mondéjar, Martínez-Martínez, Avilés, & Coy (2013) about the composition of the porcine oviductal fluid (POF). As their proteomic studies showed, this biological fluid is a complex mixt of proteins where probably many different molecular interactions are given. However, in their work, some of the proteins were emphasised for being detected in high concentration within the POF collected during the fertile window that induces zona pellucida (ZP) hardening in porcine *in vitro* matured oocytes, a prefertilization event proven to markedly

decrease the polyspermy in pigs (Coy et al., 2008a). Some of these proteins are ezrin, HSP-70-1A and HSP-90α, and they have been previously associated with different reproductive events. Therefore, it seems reasonable to think that these proteins might have an important role on ZP hardening, polyspermy prevention and/or fertilization itself. Currently, the composition of the culture media used during IVEP are either chemically defined, without any protein source, or semi defined, with bovine serum albumin (BSA) as unique source of proteins. Defined or semi defined media are preferred instead of biological fluids because, with them, major repeatability in the results is achieved. Nevertheless, omitting the addition of essential proteins present in reproductive fluids to the culture media during IVEP might have severe consequence for the efficiency of that system and even for the embryo development and later on the newborn. This fact points out to the necessity of studying the *in vitro* role of proteins identified within the reproductive organs such as ezrin, HSP-70-1A and HSP-90α on unknown aspects of the IVEP system in order to find potential oviductal and uterine factors to be included in the commercial IVF and EC media.

Secondly, the simulation of these natural fluids by including all the proteins present within them is not easy technically, posing a challenge for researchers. Moreover, the synthesis of these proteins could be very expensive. One solution to those issues, as proposed in this work, is purifying oviductal and uterine fluid collected from genital tract of sows slaughtered, where animals tracking is possible thanks to the rigorous controls carried out at the abattoir, and adding them to the culture media. This would allow the collection of reproductive fluids from females at different status of the oestrous cycle by previous visualization of the ovaries and the addition of each fluid according to their classification to different culture media. In this way, for instance, IVF media would be supplemented with the POF collected from animals around the ovulation time window, early embryo culture media with POF collected from animals at the early luteal phase of the cycle, and late embryo culture media with porcine uterine fluid (PUF) collected from animals at late luteal phase of the cycle. This approach would provide to each culture medium used *in vitro* all the molecules that take part in each event of the natural reproduction process. In addition, in pig reproduction, the most used sperm selection

method is the density gradient centrifugation using Percoll® or similar gradient media, which implies forcing spermatozoa to move along a medium whose composition is completely different to the female reproductive fluid, which could be affecting the porcine IVEP outcomes. Thus, based on the proposal of fluid simulation in this work, it was born the idea of adding reproductive fluids during sperm selection procedures as well, setting up a more natural protocol where sperm have to swim through a medium that best mimics the natural one in terms of viscosity and components.

Thirdly, the embryo culture in pigs is carried out under specific conditions of temperature and oxygen level that could be very far from those within the reproductive tract of female pigs. On the one hand, porcine IVEP is performed under atmospheric oxygen conditions (20-21 %). Nevertheless, several studies in mammals have found close to hypoxia conditions within the female reproductive tract of numerous species (Bishop, 1956; Fischer & Bavister, 1993; Kaufman & Mitchell, 1990; Mastroianni & Jones, 1965). In addition, the use of low oxygen tension during the *in vitro* culture has a beneficial developmental effect on blastocysts in mammals, which point out that atmospheric value should be no longer used (Waldenström, Engström, Hellberg, & Nilsson, 2009). On the other hand, porcine IVEP is performed at 38.5 ºC during the whole process. However, several studies in mammals have shown different temperature gradients within the reproductive tract (Bahat, Eisenbach, & Tur-Kaspa, 2005; Grinsted, Blendstrup, Andreasen, & Byskov, 1980; Grinsted, Kjer, Blendstrup, & Pedersen, 1985; Hunter, Einer-Jensen, & Greve, 2006; Hunter & Nichol, 1986). Furthermore, there are few studies assessing the effect of minimal temperature variations during IVEP despite oviduct temperature gradient is thought to play a role on directing sperm in the process of fertilization due to spermatozoa are thermo-tactile (Bahat et al., 2003). Nevertheless, this temperature gradient might have other unknown roles, which could be beneficial in *in vitro* systems. In addition, the actual oxygen and temperature reference values within the female reproductive organs *in vivo* come from previous studies where the surgical approach and probes used has been overtaken by time. Therefore, a new laparo-endoscopic single-site surgery (LESS) assisted approach is used in this work, which has been successfully employed by our research group to continuously recording pH values

within the pig oviduct (López Albors et al., 2015), along with the use of new probes. Oxygen and temperature measurements in the female reproductive tract made in this work using the pig as animal model could suggest the use of similar approaches in other species in order to improve assisted reproductive technologies (ART) outcomes.

Taking into account all the above mentioned, the approach adopted for the design of this Thesis is aimed at the understanding of physiological mechanisms influencing critical aspects of the porcine *in vitro* embryo production system by simulating *in vivo* conditions. The supplementation of culture media with exogenous proteins, detected previously within the oviduct, or directly with reproductive fluids, along with the adoption of adequate oxygen levels and temperature conditions, were the procedures selected to improve the efficiency and quality of the IVEP system in swine.

OBJECTIVES

The **first main objective** of this Thesis was to bring closer the composition of the fluid where gametes and embryos are imbibed during fertilization and development, respectively, to *in vitro* conditions by the addition of proteins previously described in the oviduct and reproductive fluids to the culture media.

The **second main objective** of this Thesis was to determine the oxygen tension and temperature conditions within the reproductive tract of female pigs in order to transfer these reference values to the porcine IVEP and to compare them with those widely used in the pig reproduction laboratories.

To achieve both main two goals of this Thesis, four **experiments** were performed:

Experiment 1. Studying the effect of previously identified proteins within the oviduct on different criteria related to porcine *in vitro* fertilization such as ZP hardening and parameters assessed after IVF.

Experiment 2. Establishing a more natural sperm selection procedure as alternative to the conventional density gradient centrifugation, and studying the use of this new procedure along with the use of a more physiological culture system by the

addition of reproductive fluids to the IVF and EC media on the outcome of the *in vitro* production of porcine embryos.

Experiment 3. Determining *in vivo* the oxygen tension within the pig reproductive tract at different reproductive status and transferring those reference values to the porcine IVEP system.

Experiment 4. Determining *in vivo* the temperature conditions within the pig reproductive tract at different reproductive status, assessing the use of these reference values during boar sperm capacitation and the porcine IVEP system.

2. LITERATURE REVIEW

2.1. A GLOBAL PERSPECTIVE ON HUMAN INFERTILITY AND ITS TREATMENTS

Human infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of having regular unprotected sexual intercourse, and it is a phenomenon affecting an increasing proportion of humanity (around 15 % of reproductive-aged couples worldwide), becoming today a global public health concern (Agarwal, Mulgund, Hamada, & Chyatte, 2015).

Unfortunately, for more than two centuries, medical science was unable to offer solutions to those couples affected by infertility due to the lack of knowledge relating to the functioning of the process of human reproduction. It was not until 1678, with the first sperm observation by the Dutch merchant and considered today as the "father of the microbiology", Antoni Van Leeuwenhoek, that the basics of reproduction began to settle, leading to the emergence of the assisted reproductive technologies (ART). Over nearly two centuries later, in 1978, the birth of Louise Joy Brown, the first "test-tube baby" took place in Oldham, United Kingdom (UK), by one of the most common ART, the *in vitro* fertilization (IVF) (Steptoe & Edwards, 1978). However, early attempts back as early as the 1890's when Walter Heape, a professor at the University of Cambridge, UK, informed the first known case of embryo transplantation in rabbits. Later, in 1959, Min Chueh Chang, a Chinese reproductive researcher, achieved rabbit live births for the first time manipulating eggs and sperm in a laboratory (reviewed by Kamel, 2013). These advances using an animal as a model were the first steps for the development of these technologies in humans.

Since the birth of Louise Brown, according to the European IVF Monitoring Programme for the European Society of Human Reproduction and Embryology (ESHRE), more than 8 million babies have been born worldwide from these procedures

(ESHRE, 2018). The progressive and unrelenting increase of infertility rates suggests that the number of babies that will be born by the use of these technologies will also grow. However, there are raising concerns about the risk of birth defects by the use of these technologies. These concerns are not only based on the short-term safety effects, such as multiple births and low birth weight (Beral, Doyle, Tan, Mason, & Campbell, 1990; Helmerhorst, Perquin, Donker, & Keirse, 2004; Jackson, Gibson, Wu, & Croughan, 2004; Reynolds, Schieve, Jeng, & Peterson, 2003; Schieve et al., 2002) but also on the long-term outcomes during adult life (Boerjan, den Daas, & Dieleman, 2000). Epidemiological studies are pointed to metabolic and cardiovascular defects such as increased blood pressure, higher fasting glucose, increased peripheral body fat deposits in childhood or adolescence, thyroid disorders, premature adrenal gland activation or advancement in female bone age. Behavioral disorders such as a potential increased prevalence of depression and attention deficit disorders have also been suggested (Duranthon & Chavatte-Palmer, 2018). Prenatal exposure to modified environmental conditions such as those provided by ART affects postnatal growth and disease susceptibility at adulthood, especially, when this circumstance is given during a long period or a susceptible time as it occurs during *in vitro* embryo production (IVEP) procedures.

Although most IVEP babies are considered healthy, the increase of studies that shows short and long-term issues is a matter of concern that is being carefully considered, as above mentioned. New researchers and procedures are aimed at reducing these risks. For example, the single blastocyst transfer is an effective method nowadays used to reduce multiple births without compromising the pregnancy outcomes (Sundhararaj et al., 2017). Differences between culture media reported by several groups focusing either on gene expression at the blastocyst stage, fetal growth, birth weight or post-natal weight during the first years of life have suggested that modifications within the culture conditions could be the key to make IVEP procedures safer (Dumoulin et al., 2010; Kleijkers et al., 2014; Mantikou et al., 2016). The majority of previous studies have been performed using small animal models such as mice. In contrast, the information coming from large animal models, with longer lifespan and physiologically closer to humans, is practically non-existent. Therefore, there is a necessity of studies focused on the

improvement of the culture conditions, especially, using animal models that most resemble human species, in order to pave the way to more safety human ART.

2.2. CURRENT STATUS OF ASSISTED REPRODUCTIVE TECHNOLOGIES IN SWINE

2.2.1. The pig as a model for translational research

Animal models are a tool already used since ancient Greece, which have become today crucial for the study of diseases and the development of appropriate treatments. Traditionally, the mouse has been a powerful experimental animal for understanding the complexity of some diseases. Mice have demonstrated biological significances in several studies. Examples of research in which knockout mice have been useful include studying and modelling different kinds of cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson's disease (Rosenthal & Brown, 2007). However, their genetic differences with humans and its size are the main limitations in medical research, making it difficult the transfer to humans of certain biotechnologies that could save many lives. In contrast to mice, the use of large animal models with higher genetic approximation to human would be required for developing these biotechnologies (Perleberg et al., 2018).

The advance in genome knowledge have revealed the high homology of pig with humans, making it an excellent biomedical model for the study and healing of human genetic pathologies (Schachtschneider et al., 2015; J. Yao, Huang, & Zhao, 2016). Moreover, its size combined with its anatomical, physiological and biochemical characteristics make of this species the main alternative to the use of other laboratory animals, contributing to not only the development of biotechnologies such as gene edition by CRISPR/Cas9, xenotransplantation, transgenesis, cloning, cellular therapy or the production of therapeutic proteins, but also to the study of global issues which need to be solved such as infertility and the concern of its treatments (Perleberg et al., 2018; Yang & Wu, 2018). For the development of the majority of these applications, the use of

reproductive technologies for producing *in vitro* porcine embryos is necessary, thus justifying the need of improved ART protocols in pigs to extend the use of this species as a biomedical model.

Furthermore, pig reproduction has an enormous economic impact in terms of animal livestock destined to food supplies since pork has been the most widely consumed and produced meat worldwide, surpassed only by poultry in 2016, according to the Food and Agriculture Organization of the United Nations (FAO) (FAOSTAT, 2016). The majority of scientists and livestock producers are fully aware that the livestock production sector is one of the major causes of the world's most pressing environmental problems, including global warming, land degradation, air and water pollution and loss of biodiversity (FAO, 2006). The development of mitigation strategies to reduce the negative impact on the planet of livestock production is imperative and necessary in order to leave a legacy for future generations. For all that, pig industry needs also to take advantage of the full range of resources and factors involved in the pig farming, making it more open and responsive to reproductive biotechnologies. These technologies are useful tools not only to improve the reproductive effectiveness of pig farms but also to try to solve one of the major urgent environmental problems.

Therefore, the importance of the use of swine for the improvement of basic and biotechnological research is indisputable in the agrarian and biomedicine fields as well as in the reproductive field. The female pig is a multiparous animal that provide a large number of oocytes facilitating the production of *in vitro* embryos on large-scale. This, along with the low development of blastocysts in this species, encourage the design of solutions for the new concerns raised regarding *in vitro* culture conditions, with a view to improve ART efficiency and reducing the diseases and problems related to them.

2.2.2. Historical evolution of reproductive biotechnology in swine

Historically, there are three major ART developed in the pig, with different levels of maturity and acceptance from the industry: artificial insemination (AI), IVEP and embryo transfer (ET). The following paragraphs will describe their historic context before

focusing this review on the current state of the different procedures taken part of the *in vitro* embryo production system in pig.

Artificial insemination

Artificial insemination was the first technology applied to improve reproduction and genetics of farm animals. It consists of the introduction of semen collected from the male into the female reproductive tract using artificial means at the proper time of the reproductive cycle of the female. The AI in swine was initiated in Russia by Ivanoff in the early 1900s (Ivanoff, 1922). Methods of semen collection and insemination were described later for this species (Christenson & Teague, 1975; McKenzie, 1931), but its development and wide commercial use in the industry did not take place until the 1980s (Brassley, 2007). Several studies related to collection, evaluation, dilution and storage of boar semen as well as detection of oestrus in sows and gilts, the timing of insemination and the insemination technique itself have improved this procedure since then. This fact makes swine AI a tool routinely used in pig farms with a high rate of success not only in terms of farrowing rates but also in litter size, being even higher than those obtained with natural mating (Roca, Parrilla, Bolarin, Martinez, & Rodriguez-Martinez, 2016). Intracervical insemination (CAI) is the standard insemination technique used, where sperm is deposited within the cervix; however, new insemination procedures based on the different deposition sites of sperm have been developed. These new protocols include semen deposition in the uterine body such as postcervical insemination or intrauterine insemination, deep in the uterine horn (deep intrauterine insemination), and in the oviduct (laparoscopic intra-oviductal insemination) (Martinez et al., 2005). These new protocols require more trained personnel but allow reducing the number of sperm per insemination, being highly effective. The boar produce a large volume of ejaculate (200 - 500 ml on average) containing around 60 x 10^9 spermatozoa during natural mating. The standard technique of CAI requires 2.5–4.0 x 10^9 spermatozoa per insemination in 80-100 ml of liquid, thus limiting the number of doses for AI that can be prepared from one semen sample to approximately 20-25 (Baker, Dziuk, & Norton, 1968). Compared to CAI, post-cervical AI allows a 3-fold reduction in the numbers of spermatozoa to be inseminated, whereas deep intrauterine AI allows up to 20-fold reduction and therefore, these two

techniques, especially the last one, increase the number of doses per ejaculate that can be prepared (Vazquez et al., 2008). This is particularly interesting when using spermatozoa of high value that are impaired.

There are different factors that have a direct influence on insemination successful rate; a clear example is sperm storage status. Despite the advantage of cryopreservation of boar semen, which allows the storage of genetic material, reestablishment of genetic lines, safeguards against environmental changes as well as different research applications (Bailey et al., 2008), 99 % of inseminations are performed with fresh or cooled semen (stored at 15 – 20 ºC up to 3 days). This is so because frozen boar sperm have low survival rate after cryopreservation and low fertility when they are used for AI (Knox, 2015). In contrast, in other domestic species such as bovine, bull frozen sperm is certainly widespread due to the satisfactory pregnancy rates. Certainly, there is a high variability in the effectiveness of the AI, being necessary to establish adequate protocols depending on the species. The speed with which the reproductive technologies are accepted and implemented by the pig industry under field conditions depends on the availability of efficient procedures. For example, sperm technologies known for many years, such as cryopreservation, and others developed later such as sperm sexing, have not been integrated into pig industry due to their limitations in terms of efficiency. Therefore, these technologies need additional studies and improvements before they can be economically profitable and applied to not only AI but also the *in vitro* production of porcine embryos, which require spermatozoa of good quality.

***In vitro* embryo production (IVEP)**

Although nowadays, AI is the leading technology used on a large scale in commercial pig breeding with most favourable cost-benefit ratio, the development of other ART such as *in vitro* embryo production is acquiring more relevance in the biomedicine and research field. Moreover, when spermatozoa of high value are severely impaired, the use of IVEP can be the best option in farms. Nevertheless, IVEP in pigs is still lagging behind in comparison with other species due to specific handicaps such as polyspermy after insemination or poor embryo development, which are more acute during

the development of these procedures in swine (Niemann & Rath, 2001). This means a greater reticence of using this technology in pig industry, which prefer the use of AI, resulting in a technological gap for porcine IVEP in comparison with other livestock species such as bovine.

Turning towards the past, the first event that led to the birth piglets by using one of the IVEP procedures was the fertilization *in vivo* of oocytes maturated *in vitro* in 1974 (Motlík & Fulka, 1974), thus establishing the IVM system. Later, it resulted in the establishment of the pig IVM-IVF system, using boar sperm capacitated in the reproductive tract, in 1978 (Iritani, Niwa, & Imai, 1978), and 6 years later, using boar sperm capacitated *in vitro* (Nagai, Niwa, & Iritani, 1984). Nevertheless, it was not until 1985, seven years after the birth of the first "tube baby", when the first piglets were born by IVF using ovulated oocytes (Cheng, 1985), and four years later, using *in vitro* matured oocytes (Mattioli, Bacci, Galeati, & Seren, 1989). While IVM-IVF system was well established, porcine embryo culture only could be carried out for a short period of time prior to the transfer at recipient females. The cause was the block of the embryo development at four-cell stage, and it was not until 1993, with improvements in the culture techniques, when embryos could be grown up to the blastocyst stage, thus, overcoming the embryonic block under *in vitro* conditions (Beckmann & Day, 1993; Petters & Wells, 1993). These entire past events have been responsible for the development of the porcine IVEP system as we know it today and efforts will be made in this PhD Thesis to overcome the current handicaps associated with this technology.

Embryo transfer (ET)

Due to the large number of offspring in sow's farrowing, the need for extra offspring per breeding female is less than, for example, in cattle breeding, where this technology is more widely extended. However, the current need for transferring pig genetic material around the world with minimal health risks and low costs, have led to a stronger motivation to use other reproductive technologies in pig industry as embryo transfer. The main advantages of embryo transfer compared to AI are, on the one hand, that 100 % of a new genome is introduced into a herd by using this technology, compared

to the 50 % by using AI and, on the other hand, the reduction of sexual transmitted diseases and the transportation costs. Safe transport of genetic material contained in an embryo from one farm to another is especially interesting for the animal welfare, reducing animal stress when embryos, instead of live animals, are transported. In addition, this way of transportation could contribute to a better acceptance of the procedures employed by animal industry in our society.

The first porcine embryo transfers were carried out under surgical procedures and general anaesthesia and it was not until 1950 when the first pigs were born by this technique (Kvasnitski, 2001). Later, endoscopic procedure for embryo transfer was developed with the advantage of a faster recovery of the animals used after this approach since a small incision was needed for the instruments (Stein-Stefani & Holtz, 1987). Nevertheless, pig ET was only used for research purposes and it had limited extent for commercial applications due to its practical limitations, relative to the use of a surgical process (Cameron, Durack, Fogarty, Putra, & McVeigh, 1989). The first experiment with non-surgical embryo transfer was reported by Polge and Day in 1968 (Polge & Day, 1968). The results of this research meant a practical progress of this technology. However, the poor transcervical accessibility of the pig uterus has been considered as major limitation in the development of non-surgical embryo transfer for a long time in this species. More than 20 years after the mentioned study of Polge & Day, five independent groups reported birth of viable piglets after non-surgical embryo transfer (Hazeleger & Kemp, 2001). One group used a sterile disposable AI-catheter (Spirette, Minitüb, Germany) in combination with a sterile disposable embryo transfer cannula (IMV, L'aigle, France), while other groups used sterile disposable AI-catheters (International Boar Semen, Eldora, USA, or FA1454, Fujihira Equipments and Instruments Co. Ltd., Tokyo, Japan). Some of these groups used large volumes of transfer medium, in contrast to the others that used small volumes. Some animals were anesthetized during the procedure while other animals were not. Although the results obtained were variable, farrowing rates were in general still low (from 9 to 60 %). As far as the 60 % figure is concerned, it should be noticed that the prenatal survival was around 16-37 % (Hazeleger, Bouwman, Noordhuizen, & Kemp, 2000; Yonemura, Fujino, Irie, & Miura, 1996). The

main limitation of this procedure was that the catheters used only could reach the uterine body and the embryos had to be deposited far from the physiological area where they should really be placed. For this reason, the design of a new technique using a catheter developed for non-surgical embryo transfer (NsDU- ET) in the ad-ovarian segment of the uterine horn of the female pig has brought a significant improvement in the widespread commercial use of porcine embryos (Martinez et al., 2013). Despite the excellent results of this new device, many factors can affect the reproductive performance of the outcomes. Among them, the effect of superovulation of the donors, the degree of synchrony between the developmental stage of the embryo and the recipients´day of cycle, the use of fresh, short-term and long-term stored embryos and the number of embryos transferred per recipient are included (Martinez et al., 2016). Before transfer, good quality embryos are selected based on their morphology under stereomicroscope because it is believed that the quality of embryos will affect the success of farrowing rates. *In vivo*-derived or *in vitro*-produced embryos can be used for this technology. Therefore, a significant improvement of the quality of *in vitro* produced porcine embryos might imply improvements and a most frequent use of ET.

2.3. *IN VITRO* EMBRYO PRODUCTION IN PIGS

2.3.1. Current situation and problems

As mentioned before, the emerging biotechnologies such as different ART, CRISPR/Cas9 gene edition, xenotransplantation, transgenesis and cloning require the use of zygotes (embryos at pronuclear stage) as well as good quality embryos at different stages of development, which might be obtained in large numbers by using *in vitro* embryo production. Because its resemblance to humans, the porcine IVEP is a useful procedure for the development of those biotechnologies. Porcine IVEP includes normally (1) the obtaining of porcine immature COCs from ovaries collected at the abattoir, (2) the *in vitro* maturation of these COCs, (3) the *in vitro* selection of boar sperm, (4) the co-culture of both gametes and (5) the culture of the resulting zygotes until the blastocyst stage (figure 1). Studies and improvements of each of those steps are critical to obtain

good quality embryos necessary for developing those biotechnologies. However, IVEP system in swine is behind, in terms of efficiency and quality, of its *in vivo* counterpart as well as of the *in vitro* systems developed in other mammalian species, slowing down the advance of those promising technologies. As already mentioned, pig IVEP is still limited by two critical factors: the high rates of polyspermy during IVF (Coy & Avilés, 2010; Funahashi & Day, 1997; Niwa, 1993; Romar et al., 2016) and the suboptimal embryo culture conditions leading to low figures of viable blastocysts for transfer (Canovas, Ross, Kelsey, & Coy, 2017; Dang-Nguyen et al., 2011; Kikuchi et al., 2002). This section looks more closely at the polyspermy and embryo culture shortcomings in this species.

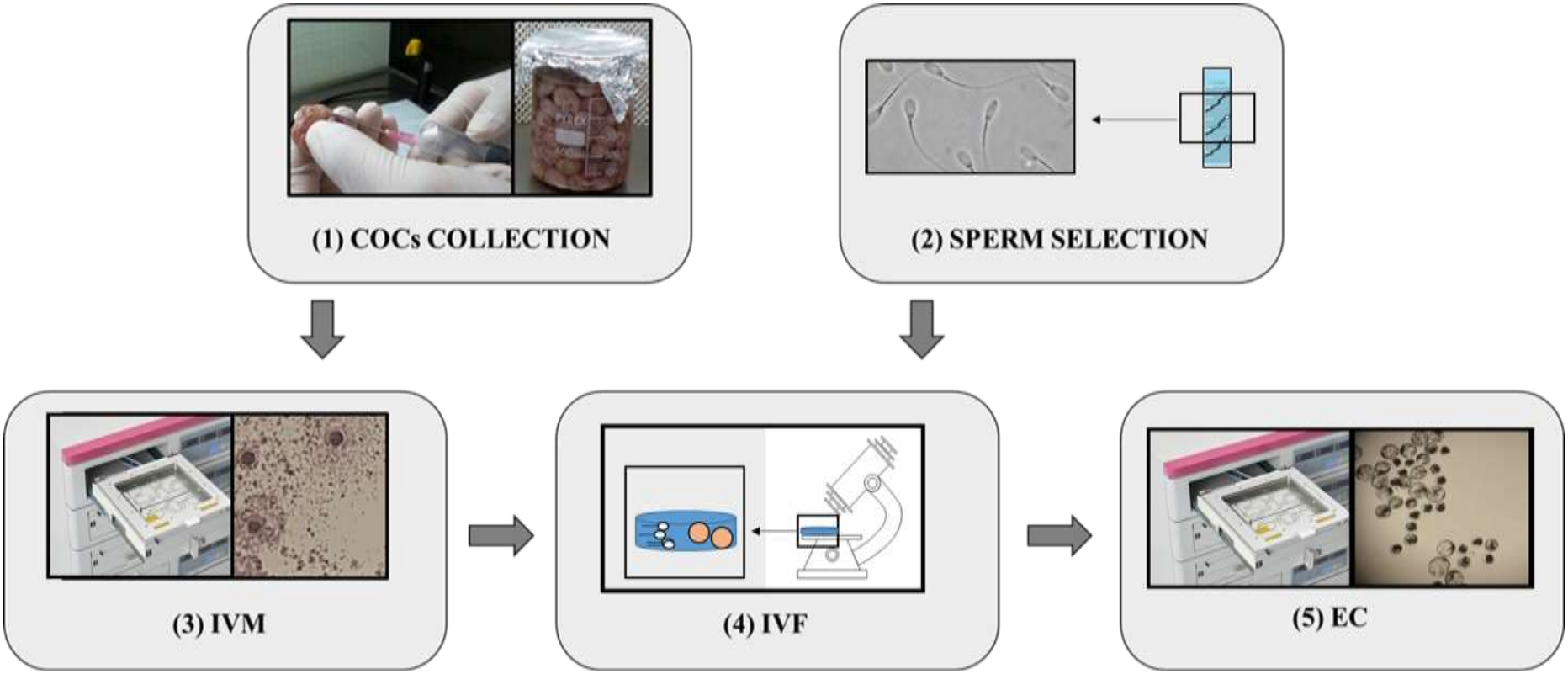

Figure 1. Schematic diagram of in vitro embryo production in swine

Polyspermy

While in swine, polyspermy is a recurring phenomenon in *in vitro* conditions, sometimes reaching 80 - 90 %, it occurs only in a 5 % of the cases *in vivo* (Hunter, 1973). High incidence of polyspermy is considered a typical and inherent characteristic of this species *in vitro* compared to other ones. Despite that fact, it was found that some of the porcine poly-pronuclear zygotes obtained *in vitro* can develop to the blastocyst stage and give live piglets, suggesting that not all pronuclei from poly-pronuclear zygotes participate in syngamy and the existence of a mechanism that allows triploid and tetraploid porcine embryos survive, even though the quality of these embryos is low (Han et al., 1999).

To explain the higher rates of polyspermy and non-viable embryos *in vitro*, it was studied, initially, how the quality of the *in vitro* matured oocytes in the pig further affects the polyspermy rates (Grupen, Nagashima, & Nottle, 1997; Wang, Sun, Hosoe, Shioya, & Day, 1997). In these studies, researchers realized that a good intercellular communication between the oocyte and cumulus cells is necessary for a competent cytoplasmic maturation and thus, the full distribution of the cortical granules that ensures an efficiency polyspermy blocking (Grupen et al., 1997; Wang et al., 1997). In order to distinguish whether this frequent issue *in vitro* is only related to an incomplete IVM, Coy et al., (1993) inseminated under *in vitro* conditions, ovulated cumulus-oocyte complexes collected from oviducts of prepubertal gilts, resulting in polyspermic fertilization as well. These studies demonstrated that biological and environmental factors during IVF seem to have a relevant impact in the polyspermy issue. Since then, polyspermy *in vitro* has been widely studied considering many biological and environmental factors, not only during IVM but also during IVF. Between them, it has been studied, for instance, the effect of biological individual variations on polyspermy rates such as those occurring using individual males with fresh and frozen sperm (Sirard, Dubuc, Bolamba, Zheng, & Coenen, 1993), between different ejaculates, and even between different fractions coming from the same ejaculate (Xu, Ding, Seth, Harbison, & Foxcroft, 1996).

Different efforts to reduce polyspermic rates by modifying the IVF protocols have been developed. The use of different sperm selection and capacitation treatments (Funahashi & Nagai, 2000; Matás et al., 2010), the adjustment of the number of sperm during each insemination (Coy et al., 1993), the adjustment of time intervals during gametes co-incubation (Coy et al., 1993), the modification of the culture media composition (Funahashi & Romar, 2004) and the modification of environmental conditions (Soriano-Úbeda, García-Vázquez, Romero-Aguirregomezcorta, & Matás, 2017) are some of the approaches used. One of the most successful procedures used *in vitro* in polyspermy blocking was the incubation of the matured *in vitro* oocytes with pre-ovulatory oviductal fluid before *in vitro* fertilization (Coy et al., 2008a). The analysis of the corresponding fluid showed that the two main responsible factors of this desired effect were one protein, Oviductin (OVGP1), together with a glycosaminoglycan, heparin (Coy et al., 2008b). This study proved the existence of a new mechanism that prevents polyspermy within the oviductal secretions by increasing the zona pellucida (ZP) resistance to enzymatic digestion and to sperm binding and penetration. Because of this, this PhD Thesis is partially focused on the use of porcine reproductive fluids and some of their components together with heparin as possible physiological factors needed to reduce polyspermy during porcine IVF.

Suboptimal *in vitro* culture conditions

Another barrier to overcome during porcine IVEP is the suitable development of the zygotes up to blastocyst stage. The rate of blastocyst formation during *in vitro* culture conditions remains low (<30 %) not only in swine but also in other mammalian species (Kidson, 2004)

Oviductal and uterine fluids share the responsibility of providing the nutrients for the development, growth and survival of zygotes and embryos *in vivo*. These fluids are composed of a combination of constituents derived from plasma and molecules secreted from the oviduct and uterine epithelium (Leese, 1988). A large number of proteins, enzymes, growth factors, cytokines and hormones have been detected within these secretions, which are assumed to have an important role during the reproductive process. However, under *in vitro* conditions, simpler culture media are used due to the difficulty

of mimicking the *in vivo* fluids composition, and embryologists are responsible for adding the adequate nutrients for the development of the zygotes and embryos to these media. After many years of study and knowledge, embryologists and researchers have designed culture media rich in many components whose concentrations have been established based on the values determined within the oviduct fluid. The main reason for that is to make an attempt of mimicking the natural environment where those embryos should develop. However, that is a difficult task still under research and culture media are insufficient for the full and adequate development of the embryos yet.

The most frequently media used for pig IVEP are North Carolina State University (NCSU) 23 medium (Petters & Wells, 1993), Beltsville Embryo Culture Medium (BECM) (Dobrinsky, Johnson, & Rath, 1996), modified synthetic oviduct fluid (SOF) (Marchal et al., 2001), NCSU-37 medium (Kikuchi et al., 2002) and porcine zygote medium (PZM) (Yoshioka, Suzuki, Tanaka, Anas, & Iwamura, 2002). The main components of these media are a balanced salt solution with an energy source, essential and non-essential amino acids and bovine serum albumin (BSA). Thanks to several researches, new requirements for embryo development have been found in the course of the history, enabling the modification and improvement of the embryo culture media. Some of the most important components that affect the embryo development are listed below:

- Energy source:

The reduction of pyruvate and lactate and the increment of glucose from the mammalian oviduct to the uterus is in consonance with the nutrient preference of the developing *in vitro* embryos (Li & Winuthayanon, 2017). Subsequent analysis of embryo metabolism revealed that glucose utilization increases during the morula stage, which suggested that this component would be essential for compaction and blastocyst formation (Sturmey & Leese, 2003). In addition, presence of glucose in embryo culture (EC) medium for the first 2 days of culture has been recognised detrimental for the development of embryos by generating Reactive Oxygen Species (ROS), which induces apoptosis in pig (Karja et al., 2006). Therefore, the development of an EC medium supplemented with pyruvate and lactate for the first 2 days and, subsequently, the use of

other EC medium with glucose as unique energetic source for the subsequent days of culture increased the proportion and quality of porcine blastocysts (Kikuchi et al., 2002).

- Aminoacids:

The use of aminoacids is also important for different events during the embryo development. Non-essential aminoacids promote the embryo compaction and the blastocoel formation while essential aminoacids are necessary for the adequate development of the inner mass of the blastocysts (Van Winkle, 2001). This is the reason why all the embryo culture media contain them to a higher or lesser extent.

- Proteins:

Proteins are one of the components more abundant in cells that provide them with their varied functions thus being of great importance. The most widely used sources of proteins in the culture media are foetal bovine serum (FBS) and BSA. The addition of these supplements to the culture media is justified because their protective effect against toxic material within the *in vitro* culture, their anti-adherent effect preventing gametes and embryos to adhere to the culture plates, pipettes or tubes and, most importantly, the provision of the requirements needed for the adequate development of the embryo. This last idea have been supported by using approaches with oviductal fluid or oviductal-cells added into the culture media, resulting beneficial for embryo development (Dubuc & Sirard, 1995; Kano, Miyano, & Kato, 1994; Kim et al., 1996). Because of the importance of proteins, this PhD Thesis is partially focused on the use of reproductive fluids and some of their contained proteins as possible physiological methods to improve the *in vitro* embryo culture outcomes.

The oviduct and uterus not only provide the nutrients but also the adequate environment for the development of the gametes and embryos. For this reason, different modifications in the physical-chemical parameters of the culture systems have been tested in order to improve the rate and quality of porcine blastocysts obtained *in vitro*. Thus, Karja et al. (2004) studied the detrimental effects of oxidative stress and how it can be effectively reduced by the application of an oxygen tension concentration closer to the one that could be found in the uterus during embryo culture in swine. Following this

strategy, two of the experiments developed in this PhD Thesis consisted of measuring the temperature and oxygen levels within the reproductive tract of the female pig and translating the reference values obtained to the porcine embryo culture system.

The study and inclusion of improvements in the culture media composition and in the physical-chemical atmosphere during porcine IVEP with the purpose of improving its efficiency is the main goal of this PhD Thesis, which pays attention to the use of conditions that best mimic those given in nature. To ensure the goal, it is then necessary to reduce the high incidence of abnormal chromosome numbers caused by polyspermic fertilization, while at the same time, the adequate culture conditions that support developmental competence of zygotes to blastocyst stage should be maintained. The latest current and new approaches that could help shape the future strategic direction of IVEP are aimed at mimicking the natural conditions found within the reproductive tract of the female pig (Romar et al., 2016). Moreover, the recent epidemiologic studies that point to an epigenetic cost in the babies obtained by the use of artificial reproductive conditions constitute a moral imperative for scientists to use more natural systems during ART (Canovas et al., 2017b; Duranthon & Chavatte-Palmer, 2018). To follow this new strategy, it is essential to identify the crucial differences occurring during fertilization and embryo development within the porcine genital tract *in vivo* in contrast with the current *in vitro* systems. These differences are reviewed below and summarized in table 1, as well as different approaches toward the use of a more physiological environment are proposed with the aim of improving the porcine *in vitro* embryo production system.

2.3.2. Differences during *in vivo versus in vitro* fertilization

In vivo, fertilization takes place inside the female reproductive tract, in the oviduct, while *in vitro* it takes place outside the living animal, in plastic culture dishes or devices and artificial conditions, deprived from the physiological mechanisms playing a role in the oviduct.

The oviduct, in addition to provide the suitable environment for the development of gametes, zygotes and early embryos, contributes to the polyspermy prevention, one of the main issues reported during porcine IVF. The main prevention mechanisms offered

by this organ, being absents *in vitro*, are based on the changes induced in the ZP of the mature oocytes by oviductal secretions (Coy et al., 2008a; 2008b) and the reduction of the simultaneous arrival of capacitated sperm to the proximity of the oocytes (Yanagimachi, 1994).

Changes induced in the ZP of the mature oocytes by oviductal secretions

In vivo, oocytes´ maturation takes place in the ovarian follicles. At the ovulation time, the ends of the oviduct or infundibulum cilia embrace the ovaries capturing the mature COCs released by a group of large follicles (ϕ > 12 mm) along with a small amount of follicular fluid (FF) (Hunter, 1977). Then, the COCs are transported to the oviduct ampulla helped by motile cilia from the oviduct ciliated epithelial cells and spontaneous contractions of the smooth muscle from this organ (Chang, 1966). This process takes about 30-45 min (Hunter, 1977) and COCs are denuded partially from the cumulus cells by means of the mechanical action that they suffer after bumping into the oviduct walls or between themselves. In the natural process of the denudation, the external glycoprotein layer of the mature oocytes, the ZP, turns into a more accessible structure to the surrounding fluid, the porcine oviductal pre-ovulatoy fluid, permitting modifications in the ZP structure of these oocytes before fertilization by different molecules present in that fluid (Ghersevich, Massa, & Zumoffen, 2015). The porcine ZP is composed of four proteins with different molecular weights and distribution: ZP1, ZP2, ZP3 and ZP4 (Dunbar, Wardrip, & Hedrick, 1980). ZP has three main roles: sperm recognition (Sacco, Subramanian, & Yurewicz, 1984), induction of acrosome reaction in sperm (Berger, Turner, Meizel, & Hedrick, 1989) and polyspermy control by the transformation of its components during the release of the cortical granules (Braden, Austin & David, 1954). In addition, during gamete interactions, changes in ZP executed by the oviductal secretions have significant implications for the sperm-ZP binding. One of the most important roles attributed to an oviductal protein, oviductin (OVPG1), is the increase in the ZP resistance to enzymatic digestion and to sperm binding, resulting in a reduction of polyspermy (Coy, Cánovas, et al., 2008). It has been shown that porcine OVGP1 penetrates two-thirds of the thickness of the zona pellucida and the presence or absence of conserved regions in the C-terminus of OVGP1 modify its association with the ZP that

affects matrix structure and renders the zona matrix permissive to sperm penetration and OVGP1 endocytosis into the egg (Algarra et al., 2016). Thus, oviductal fluid (OF) not only modifies the ZP matrix but also the internalization of some of its proteins might have a further role during embryo development.

In vitro, oocytes´ maturation takes place in culture dishes and artificial conditions and the denudation process that in natural conditions occurs in the oviduct is simulated carefully using an automatic pipette. However, oocytes are normally not exposed to oviductal secretions, unless culture media are supplemented with them. Consequently, the lack of ZP exposure to the OF and its components might be one of the main causes of the high rates of polyspermy during IVF and further deficiencies found in the *in vitro* embryo culture.

Reduction of sperm number in the proximity of the oocytes

Sperm maturation starts within the epididymis and continues during ejaculation. However, its final maturation takes place within the female reproductive tract during their ascension toward the oviductal ampulla, process known as capacitation. The capacitation involves a series of biochemical and physiological events experienced by sperm in its journey along the female reproductive tract that provide them with the necessary competences to fertilize the oocyte (sperm membrane destabilization, motility changes and acrosome reaction) (Chang, 1951). Capacitation is reversible and reproducible *in vitro*.

The female reproductive tract is the responsible of reducing the number of sperm that arrives to the fertilization place by two mechanisms:

1. Regulating capacitation status in sperm:

In vivo capacitation rates of spermatozoa reaching the oviduct are lower before than after ovulation (Smith & Yanagimachi, 1989). This seems to be due to the effect of the OF components dependent on the oestrous cycle such as the increase in glycosaminoglycan levels prior to the ovulation (Tienthai, Kjellén, Pertoft, Suzuki, &

Rodriguez-Martinez, 2000). Female reproductive tract environment, on the one hand, produces the dissociation of surface sperm glycoproteins including decapacitation factors or acrosome stabilizers that were acquired during the ejaculation. On the other hand, the loss of those factors allows the modification of sperm membrane by capacitation-inducing molecules present within this environment such as BSA, bicarbonate (HCO_3^-) and calcium (Ca^{+2}) (de Lamirande, Leclerc, & Gagnon, 1997). BSA is crucial for sperm membrane fluidity. HCO_3^- is the major ion responsible of the cyclic monophosphate adenylate /protein kinase A activation. This pathway increases tyrosine phosphorylation in many proteins and, consequently, increases sperm motility (Visconti, Krapf, de la Vega-Beltrán, Acevedo, & Darszon, 2011). Ca^{+2} is one of the molecules responsible for the changes in the sperm motility patterns and the acrosome reaction (Töpfer-Petersen, Petrounkina, & Ekhlasi-Hundrieser, 2000). In addition, a temperature gradient from cooler to warmer has been described within the porcine oviduct at ovulation time, which might play a role in sperm motility (Hunter & Nichol, 1986). The changing environment at the closest moments of fertilization increases the heterogeneous sperm population with different degrees and timing of capacitation, generating different sperm subpopulations varying in functional and fertilizing ability (Huszar & Vigue, 1993; Sousa et al., 2011). The presence of different subpopulation of sperm might be essential since each of them seems to have a different function during the fertilization process. For instance, when sperm approximate to the oocyte, a subpopulation is in charge of facilitating to another subpopulation their pass through the cumulus cells mass and the hyaluronic acid matrix that is surrounding the oocytes (Myles & Primakoff, 1997). Therefore, for the appropriate development of all these events and hence the fertilization process is necessary the existence of different sperm subpopulations with different capacitation status in charge of different functions.

In vitro capacitation can be achieved by the incubation of sperm in a simple medium that contains capacitation-supporting components such as BSA, HCO^{3-}, Ca^{+2}, an energy source and suitable temperature and pH levels. In addition, *in vitro*, sperm capacitation can be reached in a shorted time (de Lamirande et al., 1997). However, *in vitro*, defined or semi-defined culture media as well as the artificial conditions used end

up with a more homogenous sperm population with a similar capacitation pattern and a limited role during IVF.

2. Adjusting the number of sperm that reaches the fertilization place by selection mechanisms:

Tight control by natural mechanisms select a minority of the spermatozoa released at ejaculation that reach the oocytes within the female reproductive tract. Informed speculations suggest that the selection process involves mechanisms by which the quality of the fertilising spermatozoon is controlled, this way the female ensures that the offspring receives high quality genetic material (Holt & Fazeli, 2010). Within the female reproductive tract, potentially fertile spermatozoa are separated from the seminal plasma as well as from immotile and abnormal spermatozoa by active migration through the reproductive fluid and the retention of sperm by the oviductal epithelia cells before fertilization (Henkel, 2012). The first sperm screening is found in the uterus, during the extensive phase of phagocytosis, among others adverse conditions (Yanagimachi, 1994). This natural selection process makes that only a small proportion of sperm are able to arrive to the initial portion of the oviduct, the isthmus. Here, only the sperm that have not undergone the capacitation process are attached selectively to the oviductal epithelium forming a sperm reservoir (Hunter, Cook, & Poyser, 1983; Mburu, Rodriguez-Martinez, & Einarsson, 1997). The sperm reservoir seems to be mediated by carbohydrate residues such as galactosyl and mannosyl, present in the apical region of the ciliated oviductal epithelial cells, and lectin-like proteins located on the sperm head (Ekhlasi-Hundrieser et al., 2005; Suarez, 2002). This reservoir maintains spermatozoa viability and suppresses early capacitation until ovulation occurs (Ekhlasi-Hundrieser et al., 2005). Sperm release is modulated by the female oestrous cycle where an increment of this activity is observed during the periovulatory period (Suarez, 2008). Although the mechanisms responsible for sperm release are not yet known, there are different hypotheses. The first hypothesis suggests that the sperm capacitation process would be responsible of the sperm release through the loss of proteins involved in the sperm-oviduct binding or/and the increase of sperm motility (hyperactivation) (Demott & Suarez, 1992; Pacey, Davies, Warren, Barratt, & Cooke, 1995). The second hypothesis involves signalling mechanisms between

the COCs and oviductal cells (Kölle et al., 2009) together with or without the participation of hormones such as progesterone (Bureau, Bailey, & Sirard, 2002). Finally, the most recent hypothesis involves proteins detected in the oviductal fluid, which show variations along the estrous cycle (Avilés, Gutiérrez-Adán, & Coy, 2010; Mondéjar, Grullón, García-Vázquez, Romar, & Coy, 2012). The sequential release and modulation of capacitated sperm from the sperm reservoir is well known as a polyspermy prevention mechanism, which allows only a small quantity of capacitated sperm reaching the oocyte at any given moment. This could also enable the oocyte to deploy its polyspermy blocking mechanisms in an efficient manner (Hunter, 1973).

Unfortunately, *in vitro*, this important regulatory element is absent and, hence, oocytes are exposed directly to the spermatozoa. Therefore, different sperm selection procedures have been designed over the years. Such procedures prevent the penetration of abnormal spermatozoa during IVF that would normally be unable to fertilise under natural conditions. Moreover, sperm selection protocols are necessary for removing seminal plasma, which in the long term is harmful for sperm function (Martí, Pérez-Pé, Muiño-Blanco, & Cebrián-Pérez, 2006). Decades ago, sperm separation methods *in vitro* only involved one or two washing steps to eliminate seminal plasma. Then, more sophisticated techniques were developed in order to eliminate debris or dead spermatozoa and to obtain a sufficient amount of motile and functionally competent spermatozoa for IVF. These latter are classified according to their modus operandi in migration, filtration, and density gradient centrifugation procedures. However, these procedures seem to be unsatisfactory in the pig since with them the sperm population obtained has high quality but a similar capacitation status, and when they are added directly within the culture dishes next to the oocytes, all of them are able to fertilize the same oocyte resulting in polyspermic penetration.

2.3.3. Differences during *in vivo versus in vitro* embryo development

In vivo, the porcine embryo development takes place in the oviduct, the first couple of days, and in the uterus, from day 2 onwards. Both organs are responsible for maintaining the adequate nutritional requirements and conditions (pH, temperature and

gas composition) at different status of the embryo. *In vitro,* embryo development takes place outside the living animal, in culture dishes and artificial conditions, where less attention is paid to external factors such as oxygen concentration and temperature.

Timeline and early embryo development until 4-cell stage

In vivo, the first mitotic division of the porcine fertilized oocyte or zygote occurs around 18 hours after ovulation in the oviduct (Hunter, 1974). Next, embryos are maintained in the 2-cell stage for a few hours (6 - 8) and then, are kept around 20-24 hours at the 4-cell stage (Hunter, 1974). Four-cell embryos travel to the uterus 48 hours after ovulation (Hunter, 1974). The transition from maternal to zygotic control of development occurs during the 4-cell stage embryo in pig and this process is also known as embryonic genomic activation (Jarrell, Day, & Prather, 1991). This activation is a species-specific process, which occurs from 1 to 2-cell in mice, from 4 to 8-cell in pig and human and from 8 to 16-cell in cow and sheep (Li, Lu, & Dean, 2013). Before genomic activation, the embryonic development is controlled by maternal proteins and RNA, synthesized, inactivated and stored by the oocyte (Exley, Tang, McElhinny, & Warner, 1999). The transient embryo's pause within the oviduct (48 hours) prepares the uterine environment for the arrival of the embryos. It will come to the end once progesterone effects become maximal at the mid luteal phase, resulting in the dilation of the isthmus by the relaxation of the organ muscle tone and hence, the passage of the embryo to the uterus (Croxatto, 2002).

The *in vitro* development of pig zygotes to the 4-cell stage is performed under atmospheric oxygen concentration and facilitated by using a relatively simple media: a physiological salts solution supplemented with a macromolecule such as albumin and nutrients such as lactate, pyruvate, and aminoacids. Embryo development blocking at the 4-cell stage is sometimes an obstacle to *in vitro* culture and this event corresponds to the moment of embryonic genome activation (Telford, Watson, & Schultz, 1990). This tells of the sensitivity to the microenvironment of embryonic divisions at the time prior to genome activation and how embryos arrested at this stage could mean that they are low-quality embryos (Petters & Wells, 1993). One of the main limiting factors that control the

efficiency of embryo development is the quality of the oocytes and zygotes used during IVF. For example, a comparative study showed the differences between porcine embryos derived *in vivo* but fertilised *in vitro* compared to *in vivo* fertilised embryos within the overcoming of block stage (57 vs. 92%) (Rath, Niemann, & Torres, 1995). This suggest that gamete or zygote quality can be influenced by the microenvironment during IVF, so research should be focused on improving the quality of gametes and zygotes by minimizing the stressful effects on these cells. The use of culture media and physical-chemical parameters closer to the physiological conditions during IVF may seem the logical way to avoid the cell stress since they would find in a familiar atmosphere. In fact, several studies suggest that oviductal secretions (Avilés et al., 2010) or low oxygen tension (Karja et al., 2004) have a positive influence in the early embryo division rate and viability *in vitro*, becoming in factors that enhance embryo quality, however, these conditions are rarely used during IVEP.

Timeline and development from 4-cell embryo until blastocyst stage

The uterus is the organ in which porcine *in vivo* embryos will develop from the 4-cell stage until birth and where maternal-embryo communication is established (Stroband & Van der Lende, 1990). Maternal communication and the womb environment are controlled by the endocrine system as well as paracrine and autocrine signals from the embryo and the maternal tract. This complex signalling network is reflected in alteration of uterine transcriptome, leading to morphological, biochemical and immunological changes in the uterine environment. On the one hand, uterus undergoes changes throughout the estrous cycle in order to be prepared for the pregnancy moment (Skowronski, 2010). On the other hand, when embryos are present in the uterus, they also seem to be able of regulating their own uterine microenvironment by releasing steroids into the uterus since high concentrations of these hormones are found in early embryos. The mechanism suggested for maternal-embryo communication is by local vascular permeability and the release of endometrial proteins (Simmen, Baumbach, & Roberts, 1988). A number of genes and proteins have already been described to be activated in the endometrium when the embryo arrives at the maternal tract. It is apparent that precise

recognition of the embryo in the maternal tract is critical for preparation of a suitable environment for embryo development, implantation and pregnancy.

The morula stage of 8-16 cells is reached around day 4 in the uterus (Stroband & Van der Lende, 1990). Around morula stage, the compaction and mitochondria elongation are observed (Hunter, 1974), suggesting an increase in the metabolic activity of the embryo (Exley et al., 1999). Mitochondria are globularly shaped from the oocyte and the first stages of division, then they start to elongate. These organelles participate in diverse cellular processes going from the metabolism of different biomolecules to the primary energy-generating system. They are the main energy producers during different reproductive events: oocyte maturation, embryo cleavage, genome activation, compaction and blastulation and hence, mitochondrial activity is different according to the energy requirement of each stage, even being these events responsible of morphological changes in these organelles (Chan, 2006; Van Blerkom, 2009). In the compacted morula stage, the external blastomeres will start to differentiate into trophoblast, which will become part of the embryonic adjoins and the placenta; while the internal blastomeres will start to differentiate into the innner cell mass, which will become the future foetus (Johnson, 1981). During trophoblast transformation, first, the outer blastomeres form a layer of polarized cells in close contact. Secondly, binding complexes are formed between the neighbouring cells, which include tight junctions and desmosomes (Maître, 2017). The internal blastomeres will be differentiated later. Blastocyst stage of 16-32 cells is reached around day 5-6 (Hunter, 1974). This stage is characterized by blastocoel formation. The cells of the trophoblast have selective permeability, encouraging the transport of sodium and water and contributing to the formation of the blastocoel (Borland, Biggers, & Lechene, 1977), moment from which the embryo reaches the blastocyst stage. During blastocyst stage, trophoblast cells are well developed and polarized, containing a major number of mitochondria and some strands of the endoplasmic reticulum and Golgi apparatus as well as numerous microvilli oriented towards the uterine lumen; meanwhile, the cells of the internal cell mass have unequal shapes and polarity and organelles are hardly developed. Moreover, both cell types contain large quantity of lipids. Then, the blastocysts suffer a phenomenon known

as expansion, where the blastocyst takes the entire perivitelline space. The mean number of cells in the porcine expanded blastocysts range between 65-120 and the proportion of the cells mass is around 25% out the total cells of the embryo (Papaioannou & Ebert, 1988). During this stage, the ZP plays an important role in osmotic regulation. The embryo frees itself from the ZP, process known as hatching, on day 6-7 and finally, porcine blastocysts remain in the lumen of the uterus until day 13, which is a very long preimplantation period compared to that in humans or other laboratory animals (Dantzer, 1985).

In vitro, all these events are performed in culture dishes by using artificial conditions and in a relatively simple media: a physiological salts solution supplemented with a macromolecule such as albumin, and nutrients such as glucose and aminoacids. Under *in vitro* conditions, a higher prevalence of embryo developmental blocking in early stages can be noticed in many species, and always occurs at the 4-cell stage of embryo development in pig, not only coinciding with the activation of embryonic genome but also with the transit of the embryo to the uterus (Telford et al., 1990). During years, this block has been attributed to insufficient culture conditions that may prevent the activation of genes or repress the production of essential proteins, but the specific reasons of its prevalence remains unclear (Matwee, Kamaruddin, Betts, Basrur, & King, 2001). In addition, a large proportion of arrested human embryos display chromosomal aberrations (Almeida & Bolton, 1998), and that could be one of the causes of implantation failure and that European IVF pregnancy rates now steady only at around 36 % (ESHRE, 2018). In pig, less than 50 % of all *in vitro* fertilized embryos reach the blastocyst stage (Rath et al., 1995; Romar et al., 2016). Furthermore, the development of preimplantation porcine embryos able to overcome the four-cell stage is poor compared with those grown *in vivo* due to the fact that *in vitro*-produced embryos take longer to develop to blastocysts stage and contain fewer cells (<50) than their *in vivo* counterparts (65-120) (Macháty, Day, & Prather, 1998). In addition, *in vitro* produced blastocysts that develop to expanded stage may fail to hatch *in vivo* after transfer, becoming in another limiting factor for pregnancy rates (Chimote, Chimote, Nath, & Mehta, 2013). What is clear is that uterine secretions and maternal communication play an important role during this period of the embryo

development and these factors are absent *in vitro*. *In vitro* embryo culture conditions have changed little over the years and several studies point to the necessity of approaching them toward physiologic conditions that mimics the changes that occurs in nature in order to reduce the problems mentioned above (Krisher & Wheeler, 2010).

Table 1. Summary of some major differences happening within the reproductive events between *in vivo* and *in vitro* systems.

Reproductive event	*In vivo*	*In vitro*
Fertilization	- Takes place in the **oviduct** (natural conditions). - Gametes and zygotes are surrounded by a protein-rich complex medium: **oviductal fluid**, which induces changes within the oocytes' ZP and sperm. - Sperm is selected by the female reproductive tract obtaining a **heterogeneous sperm population**.	- Takes place in the **laboratory** (artificial conditions). - Gametes and zygotes are surrounded by a **simple medium**: physiological salts solution supplemented with sugars, certain aminoacids and BSA as unique protein source. - Sperm can be selected by using different methods obtaining an **homogeneous sperm population**.
Embryo development until 4-cell stage	- Takes place in the **oviduct** (natural conditions). - Embryos are surrounded by a protein-rich complex medium: **oviductal fluid**, which controls embryo gene expression.	- Takes place in the **laboratory** (artificial conditions). - Embryos are surrounded by a **simple medium**: physiological salts solution supplemented with lactate, pyruvate essential and non-essential aminoacids, and BSA as unique protein source. - Higher incidence to **block** the embryos at 4-cell stage.
Embryo development from 4-cell stage until blastocysts	- Takes place in the **uterus** (natural conditions). - Embryos are surrounded by a protein-rich complex medium: **uterine fluid**, which controls embryo gene expression. - Mean number of cells per blastocyst: **65-120** (good quality). - **Normal** growth and development of the embryos.	- Takes place in the **laboratory** (artificial conditions). - Embryos are surrounded by a **simple medium**: physiological salts solution supplemented with glucose, essential and non-essential aminoacids, and BSA as unique protein source. - Mean number of cells per blastocyst: **<50** (poor quality). - **Slow** development of the embryos and difficulty in hatching.

2.4. FUTURE IMPROVEMENTS FOR THE *IN VITRO* PRODUCTION OF PORCINE EMBRYOS

2.4.1. Addition of proteins and reproductive fluids to the pig embryo culture media

The research done during years about culture media composition helped us to identify the current nutritional needs for many types of cells. Cell culture technology has been developed in diverse areas, including not only microbiology and pharmaceutical industry but also reproductive biology. Although the cell development principles were more related to bacteria and fungus spores, the development of animal cell culture was giving rise shortly after, in 1982. This took place thanks to the development of a balanced salt solution of a composition close to that of body fluids, named by its discoverer "Ringer solution", where frog hearts beating were kept successfully after removal from the body (Ringer, 1882). After this, researchers began to pay attention to the animal culture media in order to make animal's cells grow in them. Thus, development of natural media formed by biological fluids such as plasma and blood or synthetic media that included water, salts and glucose began to be used successfully for that purpose (Carrel & Burrows, 1911). Later, synthetic culture media were significantly improved by the inclusion into them of certain complex proteins, aminoacids, vitamins and growth factors, as knowledge about cells requirements was advancing.

As mentioned previously, reproductive fluids are a complex mix of molecules, coming from transudate of blood plasma and specific oviductal or uterine secretions, which include water, salts, carbohydrates, lipids and proteins (Leese, 1988). Artificial culture media are formulated trying to imitate the composition of those fluids as close as possible by including all those components.

- Water is the major component of the reproductive fluid in which the electrolytes are dissociated participating in many reactions within the gametes, zygotes and embryos.

- Salts are dissociated within the water into electrolytes or inorganic ions such as Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, HCO_3^-, etc. In addition, to maintain the adequate pH and osmolality of gametes and embryos, many of them participate in different reproductive events (Iritani, Sato, & Nishikawa, 1974).

- Carbohydrates are added in form of sugars as pyruvate, lactate and glucose that are one of the main energy sources used by gametes and embryos (Brinster, 1965). On the other hand, complex carbohydrates, which are less used within the culture media, such as glycosaminoglycans have been related to pre-fertilization and fertilization events as well as the first stages of embryonic development (Salustri, Yanagishita, Underhill, Laurent, & Hascall, 1992; Tirone, D'Alessandris, Hascall, Siracusa, & Salustri, 1997).

- Lipids are contained in the oocyte's cytoplasm and the trophoblastic cells of the embryo and have been proposed as energy producers during oocyte maturation in species as cow and pig, with high lipid content (Sturmey & Leese, 2003) as well as precursors of steroidogenic and eicosanoid pathways to regulate physiological processes in the mammalian placenta (Han et al., 1995). Moreover, lipids such as phospholipids and cholesterol are two of the main components that form the plasmatic membrane of gametes and embryos, very important for the maturation of the gametes and other reproductive events. Furthermore, hormones commonly present within the female reproductive tract as oestrogen and progesterone are of lipid character, as well as some vitamins (vitamin A and vitamin E), which are involved in the regulation of spermatogenesis and sperm protection (de Lamirande & Gagnon, 1992; Hogarth & Griswold, 2010). Addition of lipids to the culture media, however, other than steroid hormones, is not a common practice.

- **Proteins** are one of the most important components of cells. Without them, we would not be who we are, since they are the unique molecules able to read the genome instruction and form the organisms, being responsible then, of their phenotype and proper functioning. Therefore, proteins are especially relevant during the embryo development, where embryos undergo a series of processes as they grow toward a mature organism. Numerous proteins have been found essential for the success of gametes and embryos

formation (Shin, McGuire, & Rajkovic, 2013; Standart & Minshall, 2008). However, the current global tendency is to avoid the presence of proteins in the culture media, either totally (free-protein media) or partially (media with serum albumin added). For that reason, special attention should be paid then to the impact caused by the absence of key proteins during this critical period and how the addition of these proteins to the culture media could be a solution for certain issues found *in vitro*.

One of the first complex proteins sources included within the IVF and EC media was fetal bovine serum (FBS) until it was found a positive relationship between the presence of serum in the culture media and the incidence of Large Offspring syndrome in ruminants (Young, Sinclair, & Wilmut, 1998). Nowadays, BSA replaces FBS, given that this first one is abundant within the biological fluids, in addition to having a potential impact on cell proliferation and survival (Francis, 2010). Although practical, a greater notice is taken about the impact of this approach on DNA reprogramming and fetus development. This is because the addition of BSA to the culture media does not fully mimic the protein composition of the reproductive fluids where other proteins are present as well, playing important roles on gametes pre-fertilization, fertilization and embryo development. Therefore, today, the addition of exogenous proteins to the IVF and EC culture media is another important matter of concern in the reproductive biology field for some researchers.

A recent proteomic study identified more than 1000 oviductal proteins in bovine species, and a limited number of them are specific to the stage of the estrous cycle (Papp et al., 2019). In porcine species, over 150 different proteins have been detected at nearby pre and post-fertilisation stage (Avilés et al., 2010). Some of these proteins are synthesized by the oviduct, which implies an energy cost by the animal body; therefore, should they have an important role during the different reproductive events? Some of the oviductal proteins better studied and responsible for beneficial effects on these events are osteopontin (Hao et al., 2006; Liu, Xie, Zhou, & Yang, 2015), glycodelin (Chiu et al., 2007a, 2007b), OVGP1 (Coy et al., 2008b), plasminogen (Coy et al., 2012; Mondéjar et al., 2012), Heat Shock Proteins (HSPA8, Grp78, HSP60) (Elliott et al., 2009; Lloyd, Elliott, Fazeli, Watson, & Holt, 2009), lactoferrin (Ward, Paz, & Conneely, 2005;

Zumoffen et al., 2013; Zumoffen, Massa, Caille, Munuce, & Ghersevich, 2015), Deleted in Malignant Brain Tumors 1 (Teijeiro, Roldán, & Marini, 2012), and fetuin b (Dietzel et al., 2013). *In vitro,* these proteins are not widely used in IVEP due to economic and technical reasons. However, would it be worthy the identification of proteins that participate in the reduction of polyspermy in swine for its later addition to the culture media?

In pigs, one of the most relevant effects of oviductal fluid secreted prior to ovulation on *in vitro* fertilization is its role on preventing polyspermy by increasing the required time of proteolytic digestion within the porcine matured oocyte's zona pellucida, effect known as "pre-fertilization ZP hardening". This effect has been described as a polyspermy reducing mechanism, since it regulates the entrance of sperm to the oocyte, and it has been observed *in vitro* in pigs, rabbit and other livestock species so far (Mondéjar et al., 2013). The main role for ZP hardening and monospermy increasing has been attributed to an oviductal specific protein, OVGP1, along with the glycosaminoglycan heparin (Coy et al., 2008b). However, other proteins that share certain characteristics with OVGP1 have also been identified within the oviductal fluid responsible of that hardening and thus, could we be talking about potential oviductal factors that might contribute to the ZP hardening and monospermy increase as well? Among them, ezrin and heat shock proteins such as HSP90α and HSP701A could be included (Mondéjar et al., 2013).

Ezrin is an 81-kDa protein belonging to the Ezrin-Radixin-Moesin family, consisting of three domains: an N-terminal end (FERM domain), capable of interacting with plasma membrane proteins; an intermediate domain, and a C-terminal end domain as the main actin-binding site within the protein (Bonilha, Rayborn, Saotome, McClatchey, & Hollyfield, 2006). This protein has been found within reproductive tissues, cells and fluids such as the syncytiotrophoblast of the placenta (Bretscher, Reczek, & Berryman, 1997), seminiferous tubules and Sertoli cells of testicle (Wakayama, Nakata, Kurobo, Sai, & Iseki, 2009), mouse oocyte (Larson et al., 2010), human sperm (Wang et al., 2010), compacted embryo (Liu et al., 2013; Louvet, Aghion, Santa-Maria, Mangeat, & Maro, 1996), seminal plasma (Piehl, Fischman, Hellman,

Cisale, & Miranda, 2013), and oviductal fluid (Mondéjar et al., 2013). Therefore, it has been described as a participant protein in spermatogenesis, oocyte maturation, embryo division and sperm capacitation events.

Heat Shock Proteins (HSPs), are widely spread in various mammalian species. These proteins were identified for the first time in cells after their exposure to elevate temperatures, from which their names are derived. Some members of this family are strictly induced by different stressors, not only temperature, as cell survival mechanism, and that is the reason why its production is enhanced during embryo culture conditions, whereas others are expressed at physiological conditions. Then, their functions are different depending on the circumstances under these proteins were synthesized. Under physiological conditions, these proteins act as molecular chaperones, mediating the folding and transport of other intracellular proteins and participating in the assembly of other proteins (Ellis, 1987). Under adverse circumstances including changes in temperature, the presence of free oxygen radicals, toxins, infections, etc., the mission of these proteins is the protection of the cells inducing a phenomenon known as "stress tolerance" where the cells that have suffered a previous stressful event are more prepared for a second one thus suffering less damage (Parsell & Lindquist, 1993). HSPs are classified into different families according to their molecular weight. This PhD Thesis will focus on HSP-70-1A member of HSP-70 family (70 kDa) and HSP-90α member of HSP-90 family (90 kDa) because both of them were identified in the fraction of bovine oviductal fluid with a high hardening effect on ZP (Mondéjar et al., 2013). The members of these two families are mainly located in the cytoplasm and endoplasmic reticulum. The main function of HSP-70 family is helping protein folding (assembly and refolding), especially in endoplasmic reticulum, playing a relevant role in the assembly and transport of newly synthesized proteins, whereas HSP-90 family main function is helping myosin folding and sarcomere formation. Moreover, HSP-90 is able to bind to steroid receptors, protein kinases, intermediate filaments, microtubules, and actin (Parsell & Lindquist, 1993). HSP-70 and HSP-90 members consist of three domains: an N-terminal ATP binding domain; a substrate interacting middle domain, and a C-terminal dimerization domain (Jackson, 2013). Each analogue domain differs in structure and number of amino

acids in each family. The N-terminal ATP binding domain is very important in these proteins, for instance, through ATP binding to the N-terminal domain is the way by which HSP-90 regulates muscle myofibrilogenesis (Hawkins et al., 2008). In both members several functions on fertilisation and early embryonic development have been described (Neuer et al., 2000). Thus, HSP-70 family members have been found expressed in the uterus after ovulation at the luteal phase of the reproductive cycle, and during the development of the gametes (oogenesis and spermatogenesis) and embryos, whereas HSP-90 family members have been identified during embryo development.

Despite the fact that ezrin, HSP-70-1A and HSP-90α have been characterized as possible hardening factors and they play different roles on different reproductive events (Mondéjar et al., 2013), their exogenous addition to culture media to study their impact on the hardening mechanism of the ZP and subsequent on fertilization have not been fully explored yet. Studying the effect of adding these proteins to the porcine IVF media would be of great relevance due to the current high incidence of polyspermy *in vitro* in swine. If the hypothesis of this PhD Thesis is confirmed, these proteins could be used as additives during IVF by the pig reproductive laboratories in order to take advantages of their benefits and thus, improve the efficiency of the IVEP in swine.

Reproductive secretions, present in oviduct and uterus, are the nutrient medium that provides the required molecules for the full gamete and embryo development, maintenance and function. The secretions and content of these fluids are strictly regulated by hormonal control and that is the reason why reproductive fluid composition varies throughout the female's estrous cycle (Hunter, 2012a). Chemically defined culture media used in ART attempt to mimic the composition of these fluids, with the purpose of replacing the natural environment. However, biological fluids are a complex mix of molecules very difficult and expensive to produce industrially. Thereby, there is a great concern about the damage that nutrient deficiencies in the culture media might cause to the embryo so far (Canovas et al., 2017a).

Oviductal fluid, *in vivo,* is present during the early reproductive events such as fertilisation and early embryo development, and its production is undertaken by the

oviductal ephitelial cells. *In vitro* experimental approaches have been reported giving positive effect in presence of oviductal fluid on sperm capacitation, oocyte maturation, fertilisation and early embryo development (Cebrian-Serrano et al., 2013; Coy et al., 2008; Kumaresan, Johannisson, Humblot, & Bergqvist, 2012; Lopera-Vasquez et al., 2015).

Uterine fluid found before fertilization creates a conductive environment for sperm migration and is responsible of the first screening or sperm selection whereas that found after fertilization, supports the blastocyst formation and implantation. Furthermore, extracellular vesicles and miRNA have been identified within the uterine fluid of livestock, rodents and humans, which point to an embryo-maternal communication function (Liang, Wang, & Wang, 2017). Although, most of the time, this communication involves a positive impact in the embryo development, since the embryo is able to create a response within the uterus according to its needs, occasionally the uterus can adversely affect the embryo development as it occurs for example during poor maternal dietary habits (Wood-Bradley, Barrand, Giot, & Armitage, 2015). This last could explain the observed epigenetic inheritance of maternally acquired traits, as in the case of metabolic disorders. Despite its importance, different authors have carried out several studies about the effect of oviductal fluid on the embryo development *in vitro*, but fewer are those studying the effect of uterine fluid (Hamdi et al., 2017).

Obtaining reproductive fluids (oviductal and uterine) in large amounts might be a challenge but it could provide a more realistic microenvironment for fertilisation and embryo development (Canovas et al., 2017a). Therefore, would it be the addition of reproductive fluids, sequentially, in the culture media during sperm selection, IVF and EC a major representation to those that is occurring in nature? The idea of using reproductive secretions during the IVEP should be explored under experimental conditions to unveil the relevance of these secretions for the ART outcomes.

2.4.2. Transferring of the *in vivo* parameters measurements to the *in vitro* embryo production system

To estimate environmental parameters within reproductive organs *in vivo*, care must be taken to ensure that the surgical techniques used do not damage the tissue. Important parameters that could impact the measurements include preparation of the animals before surgery, the anaesthetic protocol, the surgical approach and the biosensors used. In previous studies in pigs, where the pH and temperature were determined *in vivo*, the surgical approach to access the oviduct and uterus was by median laparotomy (Hunter & Nichol, 1986; Nichol, Hunter, & Cooke, 1997). Nowadays, the development of minimally invasive approaches based on laparoscopy allows new possibilities. This surgical procedure along with the recent development of flexible and miniaturized luminescent probes (up to 500 microns diameter), allows acquisition of more reliable data. Despite advances in surgical procedures and sensitive probes that allow accurate assessment of *in vivo* parameters, few recent studies have been conducted in mammals. In addition, no reference values of physiological oxygen exist for pig and there are not studies that assess temperature within the uterus in live mammals. Consequently, the effect of using *in vitro* physiological levels of oxygen and temperature on IVEP outcomes is still unknown.

Oxygen is the most important element in aerobic metabolism in eukaryotic cells. In addition to being an intermediary in glycolysis and oxidative phosphorylation, it regulates the generation of ROS, which are essential for the maintenance of cell signalling and homeostasis mechanisms (Thannickal & Fanburg, 2000).

In reproduction, oxygen and ROS play a critical role in events taking place in the female genital organs, such as ovulation (Shkolnik et al., 2011), sperm capacitation (de Lamirande & Gagnon, 1995), fertilization (Catt & Henman, 2000), early embryonic development and implantation (Bontekoe et al., 2012; Catt & Henman, 2000).

If we look at the *in vitro* studies, oxygen atmosphere widely used for the *in vitro* culture of mammalian gametes and embryos depends on the species. While the use of low tension (5 %) is well established in human *in vitro* culture and results in improved embryo

development and pregnancy than those using atmospheric tension (20 %), the oxygen tension is not as well standardized in animal ART. Despite several studies carried out in different species such as human, murine, bovine and even porcine have demonstrated that the use of oxygen concentrations lower than 20 % during IVEP improves developmental rates (Catt & Henman, 2000; Harvey, Kind, Pantaleon, Armstrong, & Thompson, 2004; Karja et al., 2004; Pabon, Findley, & Gibbons, 1989), IVEP in pigs normally is carried out under atmospheric oxygen conditions.

Numerous efforts to elucidate the detrimental effect on embryo development and pregnancy rates in presence of high oxygen tension compared to low tension have been made. Firstly, it has been shown that high oxygen concentrations involve an increase of ROS, incrementing DNA damage and apoptotic cells, thereby might be one of the causes in the poor quality and production of *in vitro* blastocysts (Kitagawa, Suzuki, Yoneda, & Watanabe, 2004). Secondly, it is known the existence of hypoxia-inducible factors (HIFs) produced by the trophectoderm in response to low oxygen tension, which are critical for the embryo implantation process (Bontekoe et al., 2012; Jeong, Bazer, Song, & Kim, 2016) that might be reduced *in vitro* due to the high oxygen tension, negatively affecting pregnancy rates. Furthermore, HIFs act under a low oxygen concentration environment, affecting the gene expression relative to enzymes of nitric oxide synthases type, which play an important role during sperm capacitation, and glycolytic enzymes, which are involved in embryonic metabolism (Osycka-Salut et al., 2012). HIFs also act on genes relative to vascular endothelial growth factor and insulin-like growth factor-II, which are essential on vascularization development and placentation (Harvey, Kind, & Thompson, 2007). Then, could have devastating consequences the fact of producing *in vitro* embryos under atmospheric oxygen tension and then transfer them to recipient females, where oxygen atmosphere is reduced drastically and embryos are not adapted? Studies point out that it could generate stress to the embryos and then, a harm on gene expression involving molecular and cellular mechanisms affecting the phenotype of the resulted offspring, even to next generations (Leese, Baumann, Brison, McEvoy, & Sturmey, 2008). This raises the question: why atmospheric oxygen concentration in ART is still in use? The answer

may be because of the need for studies showing the physiological oxygen concentration within the female reproductive tract.

In spite of its practical importance, not many *in vivo* studies have been carried out in mammals to obtain direct measurements of oxygen tension in organs within the female reproductive tract. Most of the available information comes from small animal models such as rat, hamster, rabbit, (Bishop, 1956; Fischer & Bavister, 1993; Kaufman & Mitchell, 1990; Mastroianni & Jones, 1965), or non-human primates such as rhesus monkey (Fischer & Bavister, 1993). No reference values have been published to date in domestic species or large animal models. Moreover, in humans, only one study reported a series of oxygen measurements within the uterus (Ottosen et al., 2006). Through analysis of the results from these previous *in vivo* experiments, it is evident that there is a wide range of values which vary depending on the species, organs, and even the physiological status of the individual (table 2). While differences in oxygen tension have been inconsistently reported in smaller animals, it is remarkable that, at least in primates, there is a drastic reduction of oxygen tension (from 8.0 – 4.6 to 1.7) experienced by the embryo as it transits from the oviduct to the uterus.

Therefore, it would be interesting to optimize the amount of oxygen necessary to maximize the efficiency of IVEP taking into account the existing amount of oxygen in the female reproductive tract according to the species, followed by the development of an adaptation system of embryos to low oxygen concentration similar to the oviduct and uterine environments. This is especially relevant in pig where there are no reference values of oxygen within the reproductive organs.

Table 2. Summary of oxygen tension within the oviduct and uterus in mammals. All studies used a polarographic microelectrode connected to a potentiometric device except Ottosen (2006) who used a luminescent oxygen probe. NA, data not available; CD, combined data from whole estrous cycle. Different letters in data of the same species refers to statistically significant differences.

Specimen	Reproductive stage	Oxygen (%)		Surgical procedure	References
		Oviduct	Uterus		
Rabbit	Late follicular	7.9	NA	Laparotomy	Mastroianni & Jones 1965
	Early luteal	7.9	NA		
	C.D (estrous cycle)	7.0	5.5	Laparotomy	

					Fischer & Bavister 1996
Rat	Proestrus	NA	4.4ab	Laparotomy	Mitchell & Yochim 1968
	Estrus	NA	3.4a		
	Metaestrus	NA	4.3 ab		
	Early diestrus	NA	6.3b		
	Late diestrus	NA	4.8 ab		
	Pseudopregnancy	NA	2.9 – 7.7		
	Pregnancy	NA	5.0 – 5.8		
	Pseudopregnant (day 4)	NA	6.4	Laparotomy	Hammer et al., 1981
Guinea pig	Early follicular	NA	5.8	Laparotomy	Garris & Mitchell 1979
	Late follicular	NA	3.3		
	Early luteal	NA	2.7		
	Mid-luteal	NA	5.1		
	Late luteal	NA	3.2		
Hamster	C.D (estrous cycle)	5.5	5.5	Laparotomy	Fischer & Bavister 1996
	Pregnancy	6.0	5.4		
	Pseudogregnancy	5.3	5.8		
Monkey reshus	Follicular	4.6a	1.7c	Laparoscopy (oviduct) & trans-cervical (uterus)	Fischer & Bavister 1996
	Early luteal	7.5b	1.7c		
	Mid-luteal	8.0b	1.7c		
Human	Late follicular to Early luteal (days 12-18)	NA	2.5	Trans-cervical (non-invasive)	Ottosen 2006

Temperature is another important regulator of the enzymatic activity of all biochemical processes, including those occurring during the conformation of an embryo. Temperature affects the transcriptome, proteome, epigenome, methylome and even the conformation of proteins, which are key event for cell function (Horowitz, 2014).

In most turtles and all species of crocodilians, ambient temperature is a deciding factor in determining the sex of the animal, and even small changes are able to modify the sex ratio (Bull, Vogt, & Bulmer, 1982; Merchant-Larios & Díaz-Hernández, 2013). Recently, it has been identified the molecular mechanism that links high temperatures during early development stages with an increment of male individuals in fish (Navarro-Martín et al., 2011). This is caused by the silencing of the aromatase gene promoter (cyp19a) through its methylation. Aromatase converts androgens into oestrogens, which are responsible for the formation of the ovaries during foetal development in all non-mammalian vertebrates. Its block due to high temperatures would therefore be the

primary cause of the testes formation instead of the ovaries. Nevertheless, what happen in mammals where embryo and foetal development is carried out inside the female reproductive tract? Has the temperature of this system a significant impact on the new-borns as it occurs for instance with ambient temperature in sex determination of certain reptiles and fish? If this is so, then, should embryologists simulate the temperature found in the reproductive organs during ART?

To answer that, firstly is important to know that mammal's core temperature is independent from atmospheric temperature and remains very stable, with ranges between 35.0 ºC to 39.0 ºC, and most of the time exceed the environment temperature. Thermoregulation is an important aspect of homeostasis. Heat is constantly generated by the body as a product of its metabolic activity and must be constantly dissipated as well. To prevent the excess of corporal heat (hyperthermia) or its deficiency (hypothermia) due to heat or cold exposition, respectively, the body has mechanisms able to relieve or generate the heat (Blatteis, 2012). This is telling us that large temperature changes might be harmful for different biological process. However, small ranges of temperature take place naturally depending of the individual status or even the age. For example, basal body temperature is diurnal, having the minimum temperature in early morning (Szymusiak, 2018). Moreover, temperature is not uniform through the body, since mammals present higher temperatures in the middle and lower in the extremities. Does it mean that minimal temperature variations have a role in normal physiological activity? If this is so, how?

In order to know the impact of minimal variations of temperature in the female reproductive tract, very few *in vivo* studies have been performed to obtain direct measurements of temperature within reproductive organs such as vagina, oviduct and ovary in different species (Bahat et al., 2005; Greve, Grøndahl, Schmidt, Hunter, & Avery, 1996; Grinsted et al., 1980; Hunter, Grøndahl, Greve, & Schmidt, 1997; Hunter & Nichol, 1986; Samples & Abrams, 1984; Yedwab, Paz, Homonnai, David, & Kraicer, 1976). Some of them are reflected in the table 3. From these studies is concluded that the temperature within the reproductive organs can vary between individuals and it normally

increases during ovulation. Moreover, a temperature gradient has been found in oviduct and ovary, more marked during ovulation time.

Despite the *in vivo* studies mentioned above, temperature widely used during ART is the body temperature, 38.5 ºC in swine, and this temperature is maintained constant during the whole IVEP. Therefore, the negative impact of not using the right temperature measured in reproductive organs according to the animal reproductive status during IVM, IVF or EC has not been well considered to date. However, recent studies show how important is the use of a temperature closer to that found in reproductive organs on ART outcome, as the work performed by Sen and Kuran (Şen & Kuran, 2018) reporting how a lower temperature (up to 2 °C) during IVM provides a suitable thermal environment for nuclear maturation and subsequent embryo development. This result is very interesting since preovulatory follicles are described to be up to 2 °C cooler than the neighbouring ovarian tissue (Grinsted et al., 1980), fact that may be essential for normal oocyte development (Aroyo, Yavin, Arav, & Roth, 2007; Grinsted et al., 1985; Wang et al., 2009). In the oviduct, the caudal region of the isthmus (sperm store site) is 0.2 – 1.7 °C cooler than the cranial portion of the ampulla (fertilization site) according to different studies (Bahat et al., 2005; Hunter & Nichol, 1986), suggesting an important effect of temperature at the fertilization time, which has not been yet assessed in IVF. The temperature value widely used during IVF in swine so far is 38.5 ºC, however, there are several studies where the temperature used has been 39.0 ºC (Abeydeera & Day, 1997; Hulinska, Martecikova, Jeseta, & Machatkova, 2011; Kikuchi et al., 2002; Koike, Matsuura, Naruse, & Funahashi, 2010; Park, Lee, Choi, & Lee, 2009; Suzuki, Saito, Kagawa, & Yang, 2003; Vatzias & Hagen, 1999; Wang, Niwa, & Okuda, 1991; Yoshioka et al., 2003).

Exposition to extreme non-physiological temperature for a longer period of time involves a remarkable damage on the future embryos during *in vivo* conditions as well as *in vitro*. For instance, *in vivo*, the prolonged exposition to high body temperatures (higher than 1.5 ºC) could negatively affect the reproductive process since compromise the testicle and ovarian function thereby affecting the developmental competence of gametes (Hansen, 2009). *In vitro*, the exposure of the gametes and embryos to temperatures equal

or higher than 40 ºC has been demonstrated to be detrimental on fertilization and embryonic development in several species (Jin et al., 2007; C. F. Silva et al., 2013). On the other hand, it has been also well studied the effect of freezing temperatures on the survival of gametes and embryos during cryopreservation procedures and, interestingly, many of the physiological effects caused by heat stress and cold stress are shared. Notable among these are the increase in the denaturation of proteins, a reduction in cell division, disruption of cellular cytoskeletal elements, changes in membrane permeability and lower protein synthesis. Moreover, cold stress can also produce alterations in the properties of the lipid bilayer and changes in the fatty acid composition of cell membranes (Sonna, Fujita, Gaffin, & Lilly, 2002).

What is perhaps less known is the effect of minor and physiological changes in temperature (equal or less than 1.5 ºC) during the reproductive process. These minimal temperature variations are given naturally in the reproductive tract for some reason and might have a beneficial effect if they are applied to the ART (Hunter, 2012b). Based on the temperature gradient in pig described by Hunter and Nichol (Hunter & Nichol, 1986) one of the strategies to be followed in order to reduce polyspermy would be the drop in temperature (up to 1.5ºC) during IVF. In a way, that change could be driving on sperm capacitated status or motility as it seems to occur *in vivo* in the isthmus achieving higher monospemic rates *in vitro* due to the reduction in the number of capacitated sperm able to fertilize the oocyte (Braden et al., 1954). Therefore, to design a work in which physiological temperature variations within the reproductive tract of female pig are determined and transferred to *in vitro* embryo production systems could be a potential improvement, deserving further research.

Table 3. Summary of temperature measured within the vagina, oviduct and ovary of mammals near ovulation period. Temperature data in oviduct and ovary were collected by invasive method (abdominal laparotomy). As a rule, the isthmus is always cooler than ampulla, and the ovarian follicles cooler than the stroma. NA, data not available.

Specimen	**Temperature (ºC)**	**Temperature gradient (absolute values)**		**Measurement probes**	**References**
	Vagina	**Oviduct**	**Ovary**		
Rabbit	NA	1.4	NA	Indwelling probes	Bahat et al., 2005
	NA	NA	2.3	Microelectrodes and/or acute thermosensing	Grinsted et al., 1980
Cow	NA	NA	1.5	Microelectrodes and/or acute thermosensing	Greve et al., 1996
Pig	NA	0.2 – 1.6	NA	Indwelling probes	Hunter & Nichol, 1986
	NA	NA	1.3 – 1.7	Microelectrodes and/or acute thermosensing	Hunter et al., 1997
Human	36.7	NA	NA	Vaginal sensor*	Yedwab et al., 1976
	36.8	NA	NA	Vaginal sensor*	Samples & Abrams, 1984

*(OvuSense ™ Warwick, UK)

3. MATERIAL AND METHODS

In this section, the materials and methods used for the different experiments conducted in this thesis are described. All the experiments were performed at the University of Murcia (Spain), except experiment 4.2 that was performed at the University of Teramo (Italy).

The experimental design overview of the whole project is depicted in figure 2 and further described at the end of this section.

Common procedures for experiments 1, 2, 3 and 4 include those related with the *in vitro* production of pig embryos and comprise the preparation of culture media, the obtaining and manipulation of biological material, the sperm selection and capacitation, the *in vitro* maturation (IVM), the IVF and the EC, as well as the assessment of different parameters related with these procedures. Additional procedures for experiments 3 and 4 include the measurement of physiological parameters by surgical approach on animals under anesthesia. All these procedures are described following.

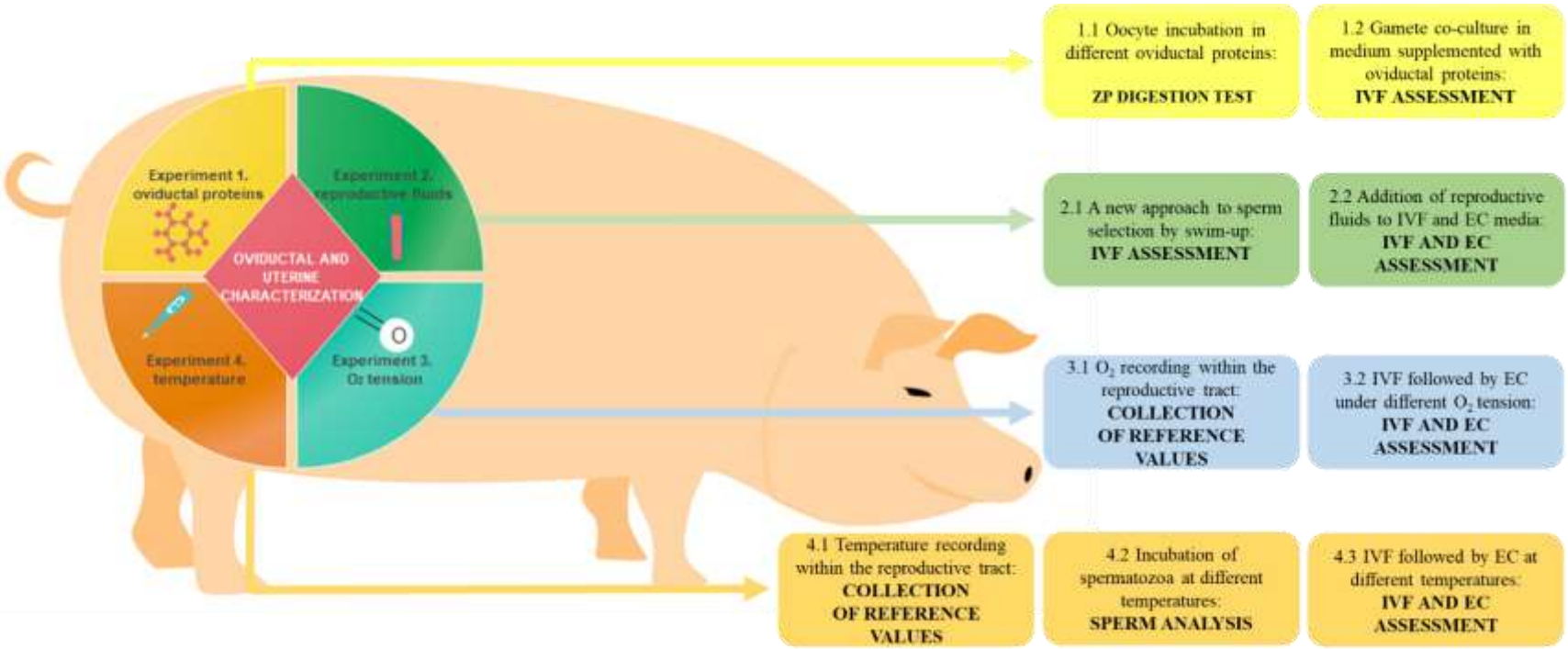

Figure 2. Experimental design. ZP (zona pellucida), IVF (*in vitro* fertilization), EC (embryo culture).

3.1. WASHING MEDIA AND CULTURE MEDIA

Unless otherwise indicated, all chemicals and reagents were acquired from Sigma-Aldrich Química S.A. (Madrid, Spain).

The preparation of these media was performed using ultrapure water produced by a MilliQ plus deionizer (Millipore Bedford, Massachussetts, USA) with an 18.2 MΩ/cm resistance. All the media were sterilized by 0.22 µm diameter filters (Millipore) under laminar flow cabinet (Telstar BH-100, Terrasa, Barcelona, Spain). Final osmolality and pH of the media were 270-320 mOsm and 7.4, respectively. For the culture media, pH was equilibrated in the incubator under 5 % CO_2 at least three hours before being used.

3.1.1. Washing media

3.1.1.1. Saline solution (SS)

Nine grams of NaCl were added to 1 liter of water. This solution (0.9 % NaCl (w/v)) was supplemented with kanamycin sulphate 100 mg/l and used to transport and wash the ovaries used during IVM procedures and to wash the genital tracts used in the fluid collection as additive of the culture media.

3.1.1.2. Cetyltrimethylammonium bromide solution (CTAB)

Zero point four grams of Cetyltrimethylammonium bromide (CTAB) were added to 1 liter of water. This solution (0.04 % CTAB (w/v) was used to wash the ovaries collected for IVM procedures and to wash the genitals tracts used in the fluid collection as additive of the culture media.

3.1.1.3. Dulbecco's phosphate-buffered saline (DPBS)

DPBS (D8662) was used supplemented with 1 mg/ml polyvinyl alcohol (PVA) (DPBS-PVA) for the handling of cumulus-oocyte complexes (COCs) for *in vitro* embryo production (IVEP) procedures and their assessment.

3.1.2. Culture media

3.1.2.1.Biological supplements for culture media

3.1.2.1.1. Porcine follicular fluid (PFF)

Ovaries from 6 months old Large-White x Landrance crossbred gilts weighing 100 kg, raised at commercial farms and slaughtered in El Pozo Alimentación S.A., (Alhama de Murcia, Murcia, Spain) were transported to the laboratory in an insulated cup containing SS at 38.5 ºC within 120 min of slaughter, washed once in CTAB and twice in SS at 38.5 ºC. The content of a varying number of follicles between 3-6 mm diameter was quickly aspirated using a 10 ml syringe connected to an 18G needle, deposited in a sterile 50 ml Falcon tube and centrifuged at 1800 g for 30 min at 4 °C to remove cellular debris. The supernatant was then filtered through 0.4 μm followed by its pass through a 0.22 μm diameter filter under laminar flow cabinet. One ml porcine FF aliquots were stored at -20 °C until their use as additives for the IVM medium for a period no longer than three months. Samples passed a quality control (pH 7.0-7.6, osmolality 280-320 mOsm/kg and a minimum 90% of Metaphase II oocytes after IVM) before their experimental use.

3.1.2.1.2. Porcine oviductal (POF) and uterine (PUF) fluids

POF and PUF (table 4) were obtained from EmbryoCloud (Murcia, Spain).

Table 4. Porcine reproductive fluids obtained from EmbryoCloud.

Name	Description
NaturARTs® POF-LF	Porcine oviductal fluid from the late follicular phase of the oestrous cycle
NaturARTs® POF-EL	Porcine oviductal fluid from the early luteal phase of the oestrous cycle
NaturARTs® PUF-LL	Porcine uterine fluid from the late luteal phase of the oestrous cycle

3.1.2.2. In vitro maturation medium

North Carolina State University (NCSU)-37 stock solution (Petters & Wells, 1993) modified by excluding bovine serum albumin (BSA) and including antibiotics, supplemented according to Funahashi et al. (Funahashi, Cantley, & Day, 1997), was used as a culture medium during IVM of immature COCs (table 5). Stock solution was kept at 4 ºC up to two weeks. Stock solution supplemented with cysteine, β mercaptoethanol and insulin was kept at 4 ºC and used within a week. PFF, hormones and dibutyryl cyclic adenosine monophosphate (dbcAMP) were added in the day of use.

Table 5. Composition of NCSU-37 stock modified from Petters and Wells (Petters & Wells, 1993) and its supplements (Funahashi et al., 1997).

Component	Concentration (mM)
Stock	
$NaHCO_3$	25.07
NaCl	108.73
KCl	4.78
KH_2PO_4	1.19
$MgSO_4•7H_2O$	1.19
$CaCl_2•2H_2O$	1.70
Glucose	5.55
Glutamine	1.00
D-Sorbitol	12.00
Penicillin G sodium	0.18
Streptomycin sulfate	0.03
Supplements	
Cysteine	0.57
β Mercaptoethanol	0.05
Insulin	0.09
PFF	10 % (v/v)
*eCG	10.00 IU/ml
*hCG	10.00 IU/ml
dbcAMP	1.00

*eCG: equine chorionicgonadotropin; *hCG: human chorionic gonadotropin

3.1.2.3. In vitro fertilization medium

Tyrode's Albumin Lactate Pyruvate (TALP) stock solution supplemented with BSA and sodium pyruvate (FERT A medium, (Rath et al., 1999) modified by replacing calcium chloride with calcium lactate as well as amikacin with kanamycin, was used as

culture medium for IVF (table 6). Stock solution was kept at 4 ºC up to two weeks. Supplements were added in the day of use.

Table 6. Composition of TALP stock and supplements modified from FERT A medium (Rath et al., 1999).

Component	Concentration (mM)
Stock	
$NaHCO_3$	25.07
NaCl	114.06
KCl	3.20
$MgCl_2 \cdot 6H_2O$	0.50
$NaH_2PO_4 \cdot H_2O$	0.35
Sodium lactate	30.00
Glucose	5.00
Caffeine	2.00
Calcium lactate	8.00
PVA	0.18
Kanamycin	0.17
Phenol red	3.00 µM
Supplements	
Sodium pyruvate	1.10
BSA	3.00 mg/ml

3.1.2.4. Embryo culture media

NCSU-23 stock solution (Petters & Wells, 1993), modified by including antibiotics and β mercaptoethanol, insulin, hypotaurine, BSA fatty acids-free (FAF) (BSA-FAF), non-essential amino acid solution (MEM ref. M7145) and essential amino acid solution (BME ref. B6766) as supplements, was named as NCSU-23B. This last one was modified by replacing glucose with sodium pyruvate and sodium lactate (NCSU-23A). NCSU-23A was used as a culture medium from zygote stage (18 hours post-insemination (hpi) until 2-4 cell embryo stage (48 hpi) (table 7). NCSU-23B was used as a culture medium from 2-4 cell embryo stage (48 hpi) until blastocyst stage (180 hpi) (table 7). Stock solutions were kept at 4 ºC up to two weeks. Stock solutions supplemented with β mercaptoethanol and insulin were kept at 4 ºC and used within a week. Hypotaurine, BSA-FAF and aminoacids MEM and BME were added in the day of use.

Table 7. Composition of NCSU-23A and NCSU-23B stocks and supplements.

Component	NCSU-23A	NCSU-23B
	Stock	**Stock**
$NaHCO_3$	25.07	25.07
NaCl	108.73	108.73
KCl	4.78	4.78
KH_2PO_4	1.19	1.19
$MgSO_4 \cdot 7H_2O$	1.19	1.19
$CaCl_2 \cdot 2H_2O$	1.70	1.70
Sodium pyruvate	0.5	-
Sodium lactate	5.00	-
Glucose	-	5.55
Glutamine	1.00	1.00
Taurine	7.00	7.00
Penicillin G sodium	0.18	0.18
Streptomycin	0.03	0.03
	Supplements	**Supplements**
β Mercaptoethanol	0.05	0.05
Insulin	0.09	0.09
Hypotaurine	5.00	5.00
BSA-FAF	4 mg/ml	4 mg/ml
100x MEM	1 % (v/v)	1 % (v/v)
50x BME	2 % (v/v)	2 % (v/v)

3.1.2.5. Sperm selection and capacitating media

Percoll® (Pharmacia, Uppsala, Sweden) density gradients of 90 % (table 8) and 45 % (table 9) (v/v) were used as sperm selection media for IVF procedures in experiments 1 and part of 2.

Table 8. Composition of 90 % Percoll® medium.

Component	Concentration (mM)
$NaHCO_3$	0.15
NaCl	0.79
KCl	1.00
$CaCl_2 \cdot 2H_2O$	1.00
$NaH_2PO_4 \cdot H_2O$	0.10
Sodium lactate	0.001
$MgCl_2 \cdot 6H_2O$	0.10
*HEPES	0.10
Percoll®	90 % (v/v)

*HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table 9. Composition of 45 % Percoll® medium consisting in 50 % Beltsville Thawing Solution (BTS) and 50 % Percoll® 90 % (v/v).

Component	Concentration (mM)
BTS	
$NaHCO_3$	15.00
KCl	5.00
*EDTA	3.36
Sodium citrate	20.00
Glucose	0.0002
Supplements	
90 % Percoll® medium	50 % (v/v)

*Ethylenediamine tetraacetic acid.

NaturARTs® PIG sperm swim-up medium (EmbryoCloud) was used as sperm selection medium for IVF procedures in experiments 2, 3 and 4.

Tissue Culture Medium 199 (TCM-199, M4530) supplemented with glucose (2.80 mM), sodium pyruvate (1.24 mM), calcium lactate (2.23 mM) and caffeine (1.00 mM) was used as sperm capacitating medium for the assessment of sperm structure and functionality in experiment 4.2.

3.2. *IN VITRO* PRODUCTION OF PIG EMBRYOS AND ASSESSMENT OF OUTCOMES

3.2.1. Cumulus-oocyte complexes (COCs) collection and selection

Ovaries from pre-pubertal Large White x Landrance crossbred gilts of around 6 months and 100 kg of weight raised at commercial farms and slaughtered in El Pozo Alimentación S.A. were transported to the laboratory in an insulated cup containing SS at 38.5 ºC within 120 min of slaughter. Then, ovaries were washed once in CTAB and twice in SS at 38.5 ºC and deposited in a beaker containing SS at 38.5 ºC on a heating plate (38.5 ºC). COCs were collected from antral follicles with 3 to 6 mm diameter by aspiration using a 10 ml syringe connected to an 18G needle. The content of the syringe was placed in a 10 ml-tube within metallic racks that allowed heat transfer during 5 min. Then, the supernatant was removed using a Pasteur pipette and 2 ml of DPBS-PVA at 38.5 ºC was added to the precipitated, which was immediately placed on Petri dishes in heating plate after its homogenization. COCs were collected using a glass Pasteur pipette

thinned at its most pointed end by heat and connected to a silicone tube (figure 3A). Only COCs with complete and dense cumulus oophorus were selected for the experiments under stereomicroscope (Nikon, Tokyo, Japan) at 20x magnification (figure 3B). COCs were washed twice with DPBS-PVA at 38.5 ºC.

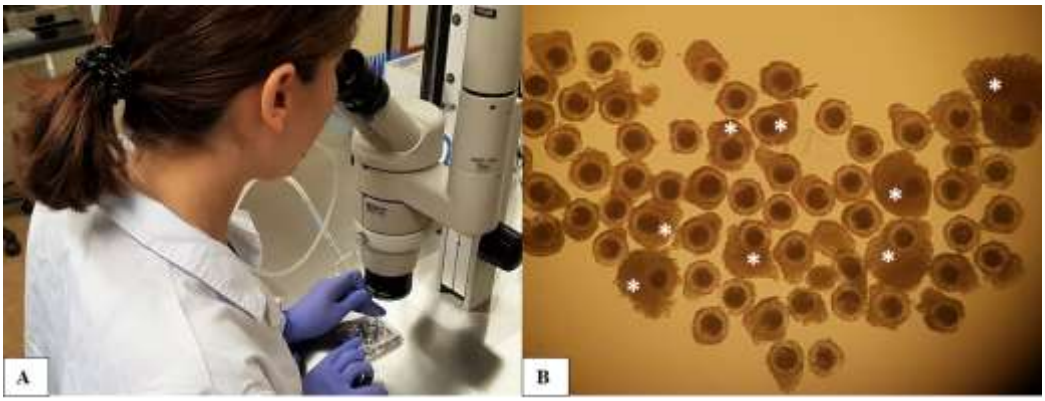

Figure 3. Selection of COCs. A: Evaluation of COCs under a stereomicroscope for the selection of the good quality complexes. B: Morphological aspect of a heterogenic sample of porcine immature COCs at 20x magnification. * Morphological characteristics of good quality COCs selected for *in vitro* embryo production.

3.2.2. *In vitro* maturation of COCs

Selected COCs were washed twice in maturation medium previously equilibrated for a minimum of 3 h at 38.5 ºC under 5 % CO_2 and 20 % O_2 in air humidity saturated atmosphere. Groups of 50 to 55 COCs were cultured in 500 µl maturation medium in four-wells multidishes (Nunc™ Thermo Fisher Scientific S.L., Madrid, Spain) for 22 h at 38.5 ºC under 5 % CO_2 in air and 20 % O_2 (experiment 1, 2 and 3) or 7 % O_2 (experiment 4) (figure 4A). Next, oocytes were washed twice in fresh maturation medium without dbcAMP, eCG and hCG, and cultured for an additional 20-22 h (Funahashi et al., 1997) (figure 4B).

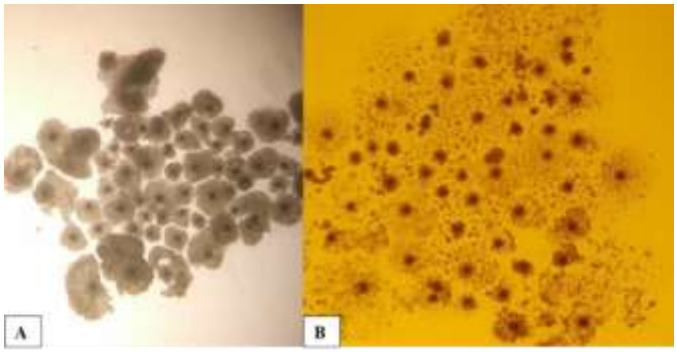

Figure 4. A: Porcine oocytes after 22 h in NCSU-37 medium with eCG, hCG and dbAMPc observed under stereomicroscope showing certain expansion of the cumulus cells (20x magnification). B: Porcine oocytes after 44 h of culture observed under stereomicroscope showing a full expansion of cumulus cells (20x magnification).

3.2.3. Semen collection, transportation and preparation

Ejaculates from Large-White, Landrace or Duroc boars of proven fertility (1-2 years old), around 200 kg of weight from CEFUSA S.A., (Pliego, Murcia, Spain) and the Experimental farm of the Veterinary Faculty at the University of Murcia (Murcia, Spain) were collected under the gloved hand technique (King & Macpherson, 1973). The first fraction of the ejaculates, poor in spermatozoa, was discarded. The second fraction of the ejaculates, rich in spermatozoa, was transported to the laboratory at room temperature (RT) and darkness within 60 min after collection.

Density gradient centrifugation using Percoll® and swim-up methods were employed depending on the experiment. Density gradient centrifugation treatment involved laying ejaculated spermatozoa on a discontinuous 45 and 90 % (v/v) Percoll® gradient (Parrish, Krogenaes, & Susko-Parrish, 1995). For that purpose, 0.5 ml undiluted semen was placed on the Percoll® density gradient media in a conical tube (figure 5A) and centrifuged at 700 g for 30 min. After that, three sperm fractions were distinguished, those spermatozoa that were not able to cross the 45 % gradient (poorly motile sperm), those spermatozoa retained between the interphase of both Percoll® gradients and those spermatozoa at the bottom of the conical tube that were able to cross the 45 and 90 % gradient (high quality sperm) (figure 5B). Then, collected high quality sperm from the bottom of the 90% fraction were washed in 10 ml TALP medium by centrifugation at 700 g for 10 min. Finally, supernatant was discarded and sperm pellet resuspended in TALP medium, sperm concentration adjusted and used immediately for insemination. Swim-up

treatment involved laying one ml of undiluted semen below 1 ml of NaturARTs® PIG sperm swim-up medium supplemented either with 0.5 % BSA or NaturARTs® POF-LF at the bottom of a conical-shaped tube using an automatic pipette (figure 6A). After 20 min of incubation at 38.5 ºC with the tube at a 45º angle (figure 6B), 0.5 ml from the top of the tube were aspirated in order to collect those spermatozoa able to swim straightly. Finally, sperm was diluted in TALP medium and used immediately for insemination, after adjusting concentration.

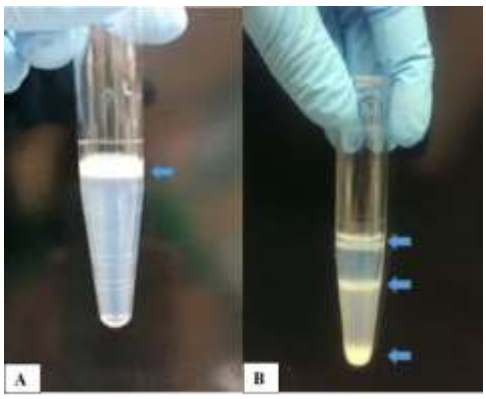

Figure 5. Percoll® treatment. A: 0.5 ml undiluted semen sample, marked with a blue arrow, placed on the top of Percoll® density gradient media. B: After centrifugation, the different sperm fractions, marked with a blue arrow, were visualized. At the bottom of the tube, the spermatozoa fraction used for insemination was available.

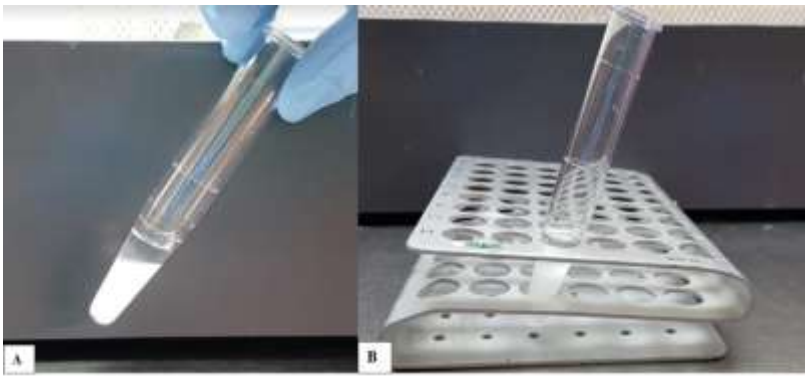

Figure 6. Swim-up protocol. A: 0.5 ml undiluted semen placed at the bottom of a conical tube with 1 ml NaturARTs® PIG sperm swim-up medium. B: the tube was placed forming an angle of 45 º during sperm incubation.

3.2.4. *In vitro* fertilization (IVF)

After 44 h post-maturation, cumulus cells were partially removed mechanically by pipetting. Next, partially denuded porcine oocytes were washed twice in TALP medium. Finally, oocytes were distributed in groups of 50 to 55 in four-wells multidishes containing 250 µl TALP medium, and 250 µl TALP medium containing sperm were added at a ratio of 1,000 to 2,000 spermatozoa per oocyte. Doing so, spermatozoa and oocytes were co-incubated in a final volume of 500 µl TALP medium. Additives of the IVF medium, oxygen and temperature were modified depending of the experiment.

Eighteen hpi, putative zygotes were transferred to embryo culture medium. A sample of 30-50 putative zygotes from each group was taken for assessing fertilization rates.

3.2.5. Embryo culture (EC)

At 18 hpi, putative zygotes were washed and transferred to four-well dishes containing 500 µl NCSU-23A medium. After 48 hpi, only the 2-4 cell embryos were washed and transferred to 500 µl NCSU-23B medium for the rest of the culture period (up to 180 hpi). Additives of the EC media, oxygen and temperature were modified depending of the experiment.

3.2.6. Assessment of *in vitro* fertilization outcome

At 18 hpi, putative zygotes were transferred to DPBS-PVA at RT and the rest of cumulus cells and sperm not bound to the ZP were removed by pipetting. Next, putative zygotes were fixed for 30 min with 0.5 % glutaraldehyde in DPBS-PVA followed by DNA staining in 1 % (w/v) Hoechst 33342 in DPBS-PVA during 20 min in the dark at 4 ºC. Hereafter, the putative zygotes were washed in DPBS-PVA at RT during 5 min and then transferred in groups of 25 to a 10 µl drop of mounting medium, consisting of 66.4 % DPBS-PVA (v/v), 33.3 % glycerol (v/v) and 0.3 % Hoechst 33342 (v/v) on a slide covered with a coverslide. Putative zygotes were examined under epifluorescence microscopy (Leica® DMR, USA) at 400x and at wave length of about 495 nm to assess the fertilization rates.

The parameters assessed after IVF were the following:

- Percentage of penetrated oocytes (**penetration %**): number of oocytes penetrated per one or more sperm out of the total inseminated oocytes.

- Percentage of monospermic oocytes (**monospermy %**): number of oocytes penetrated by a single sperm out of the total penetrated oocytes.

- Mean number of spermatozoa per oocyte (**S/O**): mean number of spermatozoa per penetrated oocyte.

- Percentage of male pronucleous formation (**MPN %**): number of oocytes, with, at least, one male pronucleous, out of the total penetrated oocytes.

- Mean number of spermatozoa bound to the zona pellucida (**ZPb**): mean number of spermatozoa attached to the zona pellucida of the total penetrated oocytes.

- **IVF yield (%)**: it was calculated as the number of zygotes showing only two pronuclei out of the total inseminated oocytes.

Immature oocytes at nuclear level were discarded from the study such as oocytes at germinal vesicle stage (figure 7A) or metaphase I (oocytes showing metaphase plate without extrusion of first polar body). Those oocytes at metaphase II (metaphase plate and extruded first polar body) without spermatozoa inside the ooplasm were considered as mature oocytes but non penetrated (figure 7B). All the spermatozoa penetrated in the matured oocytes were recorded, indistinctly of the different degrees of chromatin decondensation: compact, partially decondensed chromatin (figure 7D) or at the pronuclear stage (figures 7C and 7D). On the other hand, it was necessary to find two pronuclei inside the penetrated oocytes to consider that these oocytes had the ability to form male pronucleus, since one of the pronuclei would correspond to the female one. The sperm bound to the ZP were distinguished from the sperm within the oolema by the optical level provided by the fine adjustment knob of the microscope and the combined use of bright field observation together with fluorescence filter, which permitted to observe the sperm tail in those bound to the ZP (figure 8).

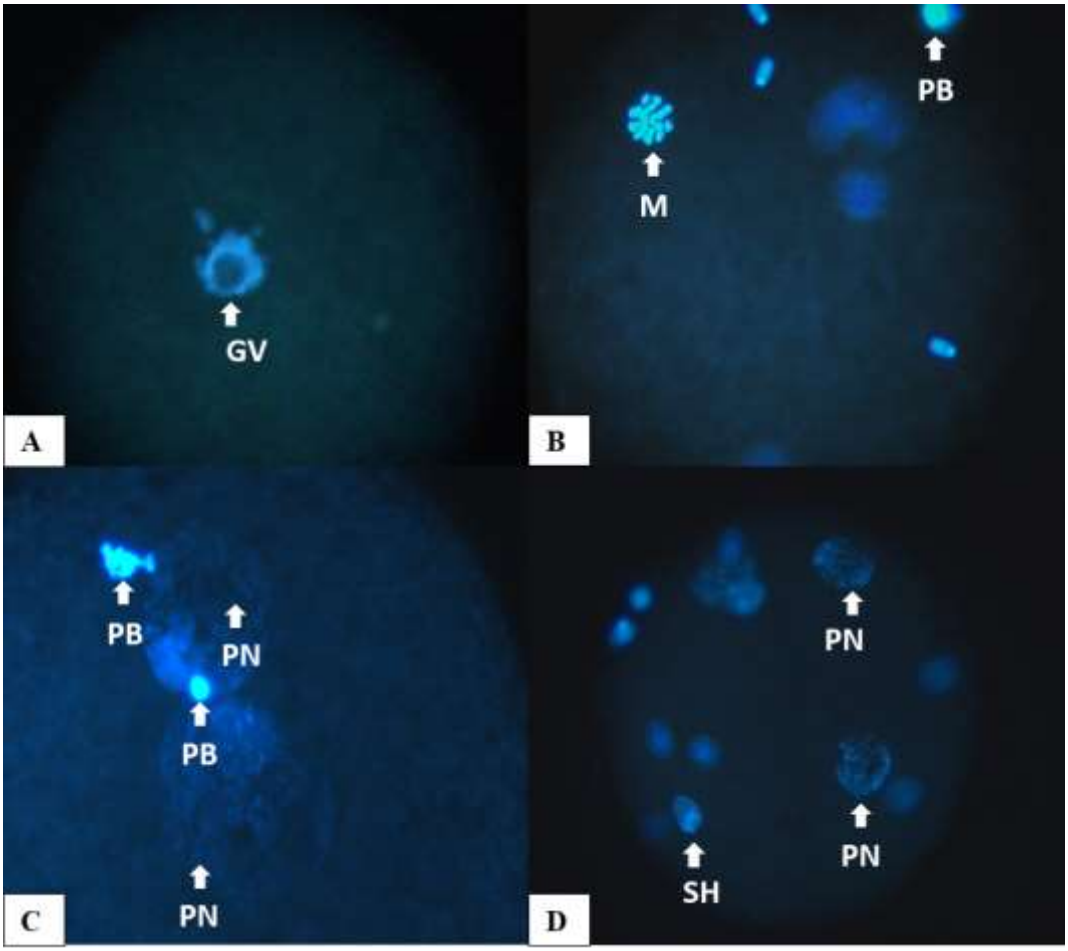

Figure 7. Images of fluorescence microscopy (400x) showing porcine oocytes stained with Hoechst 33342 in different nuclear stages. A: Immature oocyte at germinal vesicle stage (GV). B: Mature no penetrated oocyte with metaphase II plate (M) and polar body (PB). C: Oocyte penetrated by a single spermatozoon (monospermic oocyte). Male and female pronuclei (PN) have been able to develop, and also the extrusion of the second polar body (PB), which occurs after fertilization, is visible. D: Oocyte penetrated at least by two spermatozoa (polyspermic oocyte); one of the sperm heads (SH) showed partially decondensed chromatin but was not able to form the male pronucleus.

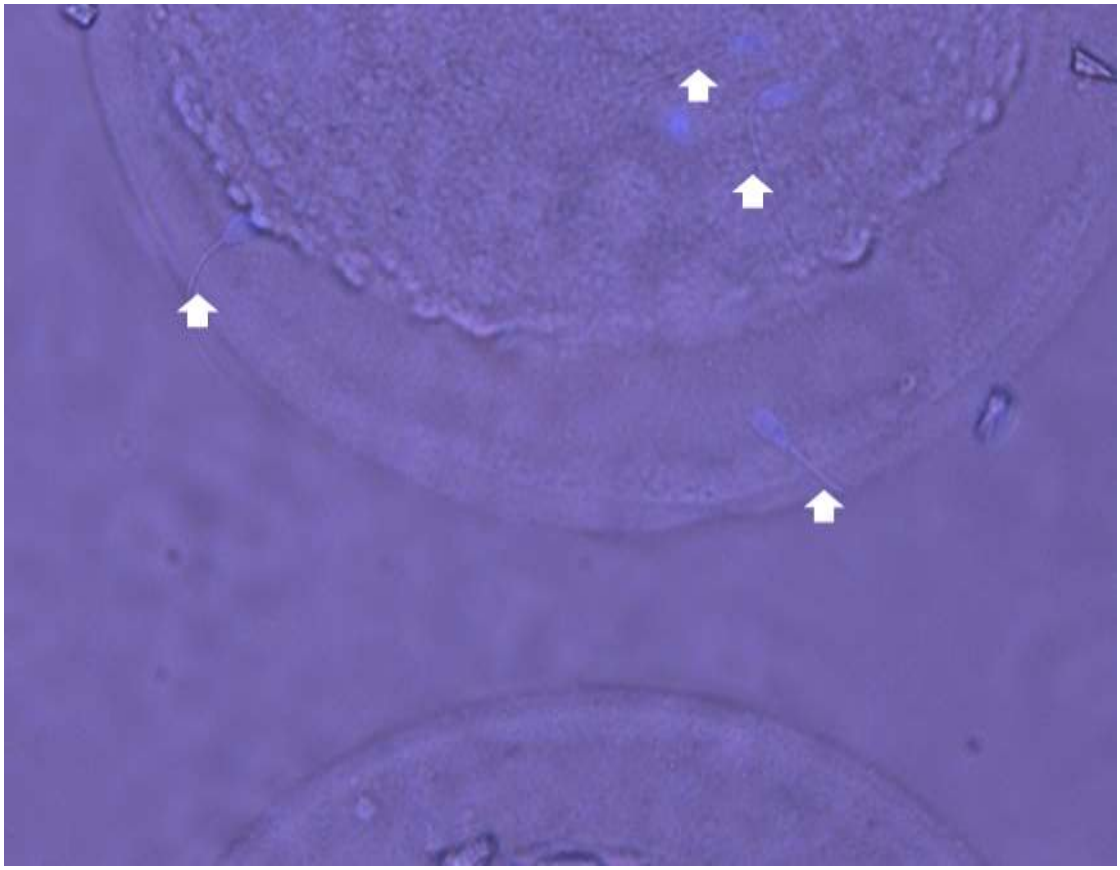

Figure 8. Image taken by combination of bright field together with fluorescence microscopy (400x) showing a porcine oocyte stained with Hoechst 33342. Arrows indicate the tail of the sperm bound to the ZP.

3.2.7. Assessment of embryo culture outcome

The parameters studied during EC were the following:

- The percentage of embryonic division (**cleavage %**) was assessed under the stereomicroscope at 63x and it was calculated as the percentage of 2-4 cell embryos out of the total putative zygotes at 48 hpi.

- The percentage of blastocyst (**blastocysts %**) was assessed under the stereomicroscope at 63x and it was calculated as the percentage of embryos reaching the blastocyst stage out of the total of 2-4 cell embryos as well as the percentage of embryos reaching the blastocyst stage out of the total of oocytes, both at 180 hpi.

- **Stage of blastocyst development** and **morphological quality of the blastocysts**: these parameters were assessed individually for each blastocyst under the

stereomicroscope at 63x on day 7.5 (180 hpi). Criteria for the classification of the blastocysts in these two parameters, modified from Bo and Mapletof (Bo & Mapletof, 2013), were as follows:

Developmental stages (figure 9):

- Early blastocyst (Stage 5): embryos that had formed a cavity or blastocoel and occupied up to 80 % of the perivitelline space. It was difficult to differentiate inner cell mass from trophoblast cells at this stage of development (figure 9A).
- Blastocyst (Stage 6): embryos with a highly prominent blastocoel occupying whole perivitelline space. Visual differentiation between the trophoblast and the inner cell mass was possible at this stage of development (figure 9B).
- Expanded blastocyst (Stage 7): embryos that dramatically increased their overall diameter with a remarkable thinning of the ZP to approximately one-third of its original thickness (figure 9C).
- Hatching blastocyst (Stage 8): embryos that were able to begin hatching from its ZP (figure 9D).
- Hatched blastocyst (Stage 9): embryos that had completely shed the ZP. Hatched blastocysts were often spherical with an inner cell mass and trophoblast well defined (figure 9E).

Morphological quality:

- Code A (Excellent or good): embryos that had uniform blastomeres in size, color, and density and less than 20 % extruded material in the perivitelline space.
- Code B (Fair): embryos that might have blastomeres with small differences in size, color, or density and less than 50 % extruded material in the perivitelline space.
- Code C (Poor): embryos that often had blastomeres with great differences in size, color, or density and more than 50 % extruded material in the perivitelline space.
- Code D (Dead or degenerating): non-viable embryos, oocytes or 1-cell embryos that do not reach the blastocyst stage.

- Mean number of cells per blastocyst (**cells/blastocyst**) was assessed under the epifluorescence microscopy at 400x after staining with Hoechst 33342 following the protocol described for zygotes staining. Each blastocyst was individually mounted on the slide; this allowed the association of their morphological characteristics in fresh (stage of development and quality) with the mean number of cells (figures 10A and 10B). Cells/blastocyst was calculated by the mean number of cell counted per blastocyst.

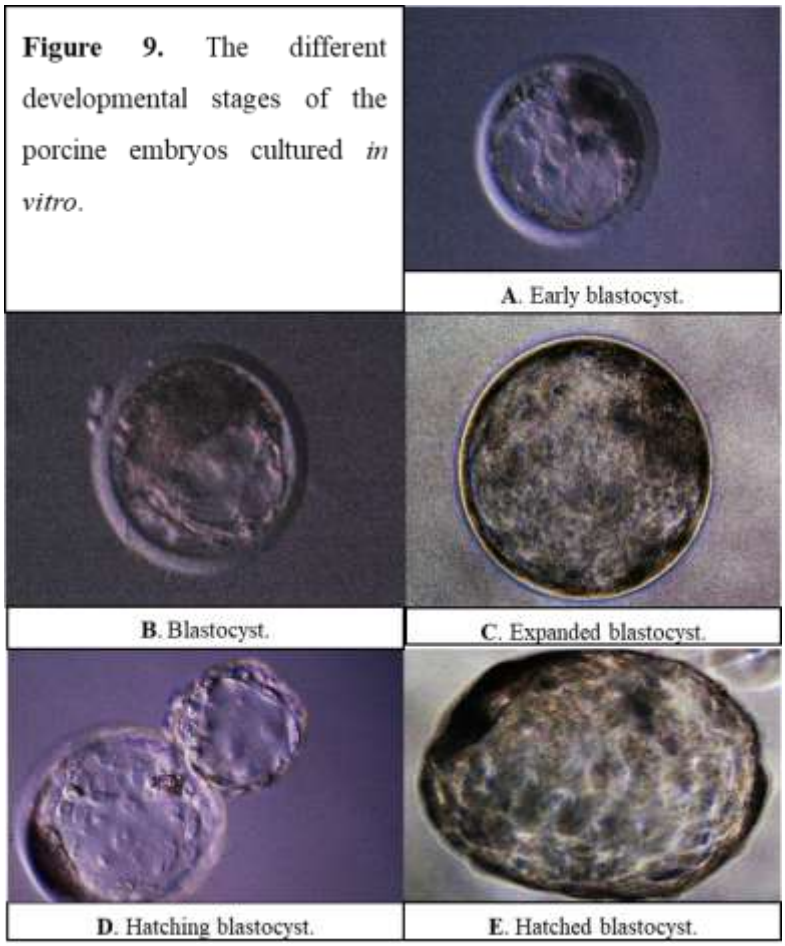

Figure 9. The different developmental stages of the porcine embryos cultured in vitro.

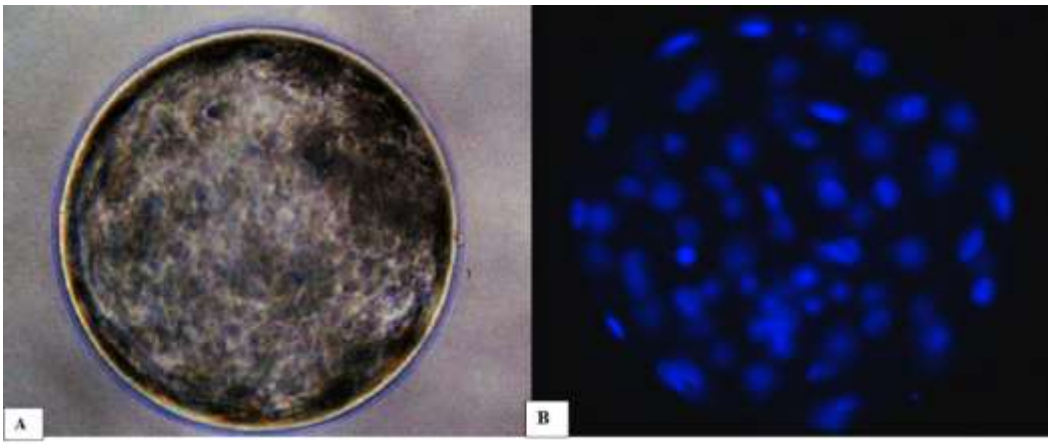

Figure 10. A: Image of a single fresh porcine blastocyst on day 7.5, classified as 7A, obtained from an inverted microscope at 40x magnification. B: Image of the same porcine blastocyst after staining, obtained from an epifluorescence microscopy at 40x magnification. This blastocyst count with 74 cells.

3.2.8. Collection of blastocysts *in vivo*

Large White sows 1-2 years old, around 150 kg of weight, weaned 28 days after parturition, and showing sign of estrous 5 days later, were inseminated in the collaborative farm CEFUSA S.A., and slaughtered in El Pozo Alimentación S.A. 7.5 days after insemination. Genital tracts were collected and transported to the laboratory in a thermoflask where uterine horns were briefly dissected and washed once in 38.5 ºC tempered CTAB and twice with DPBS-PVA at RT within 120 min from slaughtering. The washes were performed by introducing 20 ml of tempered DPBS-PVA within the distal end (utero-tubal junction) of each horn using a syringe connected to an 18G needle; then, gently, a manual pressure gradient from there to the proximal end of the uterine horn letting the fluid drop into a 50 ml Falcon tube was made. Blastocysts were identified under the stereomicroscope, collected and stained with Hoechst 33342 following the previous protocol for cell counting.

3.2.9. Assessment of zona pellucida (ZP) solubility

After 44 h post-maturation, cumulus cells of oocytes were completely removed mechanically by pipetting and oocytes were incubated in TALP medium according to experiment 1.1, and then, washed twice again in TALP medium. The oocytes were then washed in DPBS-PVA at 38.5 ºC and transferred into drops of 50 µl 0.5% pronase (w/v,

protease of *Streptomyces griseus* in DPBS-PVA) covered by mineral oil (Coy, Gadea, Romar, Matás, & García, 2002). ZPs were continuously observed for dissolution under an inverted microscope equipped with a warm plate at 38.5ºC. The dissolution time of the ZP of each oocyte was registered as the time between the placement of the oocytes in the pronase solution (time 0) (figure 11A) and the time when ZP was no longer visible at 40x (figure 11B). This time was referred to as ‘‘ZP digestion time’’ (Coy et al., 2002).

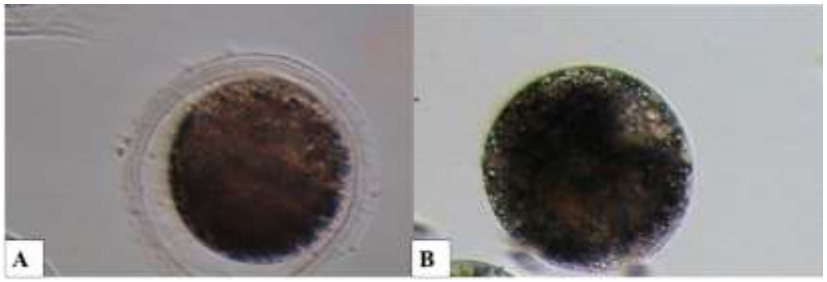

Figure 11. Image of an *in vitro* matured porcine oocyte obtained from a microscope at 40x magnification. A: At time 0, the oocyte has intact ZP. B: At the ZP digestion time, the ZP is no longer visible.

3.2.10. Assessment of sperm structure and functionality

Boar sperm doses purchased at Inseme s.p.a. (Modena, Italy) were washed twice in DPBS-PVA by centrifugation (10 min at 850 g) (Barboni et al., 2011). Then, washed spermatozoa were incubated in Petri dishes containing 2 ml TCM 199 medium during 2 hours under 5 % CO_2 and 20 % O_2 in humidified atmosphere at 38.5 ºC for the sperm membrane structure analysis and at three different temperatures (37.0, 38.5 and 39.5 ºC) for the membrane fluidity analysis. The final sperm concentration used was 1 x 10^8/ml.

3.2.10.1. Sperm membrane structure

After incubation, sperm samples were washed in DPBS-PVA by centrifugation (10 min, 4 ºC at 850 g), followed by the membrane extraction. Therefore, sperm was suspended in hypotonic buffer (2 mM Tris, pH 7.2, 12 mM NaCl) and sonicated 6 times for 15 sec, each pulse separated by 1 minute at 70 % power and 4 ºC. After a low speed centrifugation (15 min, 4 ºC at 1100 g), samples were suspended in a potassium phosphate buffer (1 mM potassium phosphate, 250 mM sucrose, 0.1 mM EDTA). Each sample was resuspended and centrifuged in the same buffer three times and the supernatants pooled

and centrifuged at high-speed centrifugation (100.000 g, 4 ºC for 1 h). The pellet of this last centrifugation, containing the membrane enriched fraction, from 6 x 10^8 spermatozoa, was lyophilized at -20 ºC and 0.3 hPa for 24 h and stored at RT protected from light. Shortly before analysis with the differential scanning calorimetry (DSC) the pellet was weighted (average sample mass was 2-5 mg) and suspended in double volume (w/v) of distilled water. DSC was performed with a calorimeter (Perkin Elmer DSC-7, Milano, Italy). Scans were performed at 10 ºC/min in a range from 10 to 90 ºC.

3.2.10.2. Sperm membrane fluidity

Membrane fluidity was assessed using lipophilic fluorescent molecule DilC12(3) perchlorate (ENZ-52206, Enzo Life Sciences, USA). During the last 15 min of sperm incubation, 2 µl of DilC12(3) stock solution (100 mg/ml) were added to the Petri dishes containing 2 ml TCM-199 medium and the spermatozoa. Fluorescence recovery after photobleaching (FRAP) was performed in at least 10 spermatozoa per group, with a confocal microscope (Nikon A1r laser confocal scanning microscope, Tokyo, Japan) equipped with the NIS-Element software, using a Plan Apo λ 100x Oil objective (numerical aperture: 1.45; zoom: 1x; refractive index: 1.515; pinhole size: 69 µm; 1 picture every 0.512 sec). Fluorescence bleaching and recovery were conducted as follows: λexc = 561.5 nm; λem = 595/50 nm with 1 scan for basal fluorescence record at 2.4% of the maximum laser power, 1 scan at 100% laser power for bleaching, and 25 scans for monitoring recovery at 2.4% of the maximum laser power. Recovery curves were realized and analyzed by using the simFRAP plug-in for Fiji ImageJ (https://imagej.nih.gov/ij/plugins/sim-frap/index.html) (Blumenthal, Goldstien, Edidin, & Gheber, 2015) computing the diffusion coefficients of fluorescent dye embedded in cell membrane, regardless of bleaching geometry. The algorithm was based on fitting a computer-simulated recovery to actual recovery data of a FRAP series. The requested parameters were set as follow: pixel size: 0.12 µm; acquisition time per frame: 0.12 sec. The results were expressed as calculated diffusion coefficient (CDC) (cm^2/sec).

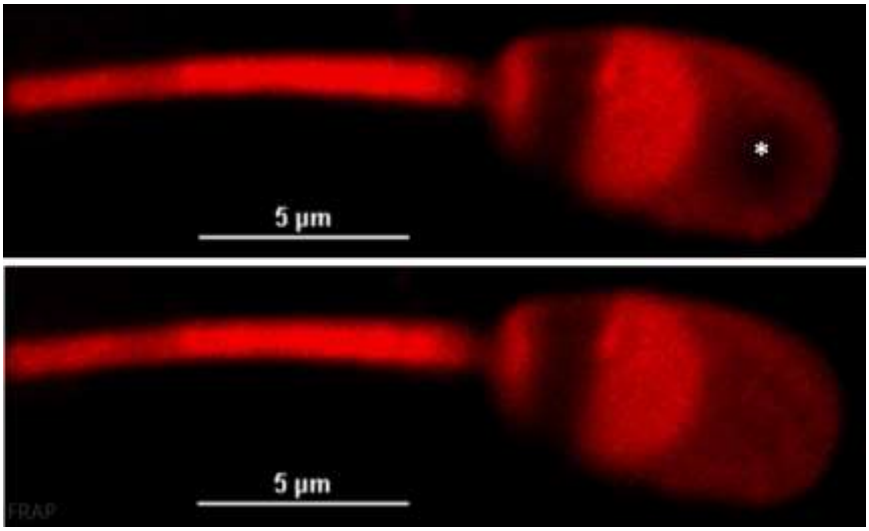

Figure 12. Spermatozoon stained with DilC12(3) observed under a confocal microscope using a 100x oil objective. Above, 1 scan at 100% laser power for bleaching, * indicate the place where the bleaching was performed. Below, the last scan after recovery at 2.4% of the maximum laser power.

3.3. *IN VIVO* MEASUREMENTS OF OXYGEN AND TEMPERATURE IN THE REPRODUCTIVE TRACT OF LIVE ANIMALS

3.3.1. Ethics

The experimental work with animals was carried out following guidelines of the Spanish Policy for Animal Protection RD 53/2013, which meets European Union Directive 2010/63/UE of the European Parliament on animal protection law. All the procedures performed were approved by the ethical committee of the University of Murcia and by the Animal Production Service of Agriculture Department of the Region de Murcia (Spain) (ref. CEEA-OH 192/2017).

3.3.2. Animals

For experiments 3.1 and 4.1 (see figure 1 and experimental design at the end of this section) pigs of mixed Large-White x Landrace breed were randomly selected from the stock kept in the animal facilities of the Experimental farm of the Veterinary Faculty at the University of Murcia. Gilts and sows were used. Gilts had an approximate age of 5 months and weighed 80-117 kg (average 95 kg). Gilts were induced to ovulate with a

single dose of 1000-1500 IU equine chorionic gonadotropin (eCG) (Foligon; Intervet International BV, Boxmeer, Holland) injection, based on their weight, followed by a 750 IU hCG (Veterin Corion; Divasa Farmavic, Barcelona, Spain) injection, 56 hours after eCG (Coy et al., 1993). The expected time for ovulation is 38-42 h after hCG, and during this period, the gilts showed external and behavioral characteristic of estrus such as rigid stance (lordosis reflex) when back pressure was applied, swollen and reddish vulva with mucous discharge, depressed appetite, restlessness, alertness or grunting. Sows were aged 24-48 months, weighed 140-280 kg (185 kg average) and had at least three pregnancies before being used in these experiments and, after weaning, their sexual behaviour was confirmed using a mature fertility-tested boar. Pigs had *ad libitum* access to water, were fed with commercial food appropriate for their productive state, and were kept under a natural photoperiod with a temperature range of 16-27 ºC.

3.3.3. Anaesthesia

Pigs were kept in individual pens under fasting conditions 24 hours before surgery. The day of surgery, animals were sedated with atropine (0.04 mg/kg), diazepam (0.4 mg/kg) and ketamine (10 mg/kg) followed by propofol (3 mg/kg), all via intramuscular. Once pigs were completely sedated, they were placed on the surgical table, intubated and mechanically ventilated. Anesthesia was maintained with isofluorane (2-3 % O_2) and intravenous perfusion of 0.9 % sodium chloride (2 ml/kg/h).

3.3.4. Surgical procedure

Pigs were disposed in lateral *decubitus* position and gas insufflation (pneumoperitoneum) was induced with 8-10 mmHg CO_2 in order to provide sufficient space for surgery and ensure adequate visualization of the structures and manipulation of the instruments. Hereafter, a paralumbar LESS assisted approach was carried out with a monoport device (GelPOINT® Advanced Access Platform, Applied Medical, Rancho Santa Margarita, California, USA) (Figure 13). This approach consisted of: 1) the surgical incision of 5 cm approximately (mini-lumbotomy) on oblique major and minor muscles and transverse muscle of abdomen using a scalpel (Figure 13A). 2) The placement of the monoport device composed by two flexible rings connected by a plastic membrane (figure

13B) and the monoport cap. One of the rings was placed in the abdominal cavity, the other laying on the external incision, and both of the rings were used to stabilize the incision in the abdominal wall (Figure 13C) and insert the monoport cap. The monoport cap had entrance for three ports (Figure 13D). Three laparoscopy ports were made through the gel of the monoport cap, one of the ports was used for the insertion of the laparoscope to the abdominal cavity and the other two for the insertion of instruments to hold the organs. 3) The visualization of the abdominal organs up to holding of the ad-ovarian end of the uterine horn towards the port opening using surgical forceps (non-traumatic) (Figure 13E). 4) The removal of pneumoperitoneum and the monoport cap to allow the manipulation of the reproductive organs (ovary, oviduct and uterus) (Figure 13F). 5) The classification of the pigs' reproductive stage based on the ovaries visual inspection in early follicular (EF), peri-ovulatory (PO) or luteal (LU) phase. Specific criteria for categorization included: EF, pigs whose ovaries contained only follicles 1-2 mm in diameter and less than 6 follicles of 8-12 mm in diameter; PO, pigs whose ovaries contained more than 6 follicles of 8-12 mm in diameter or more than 6 hemorrhagic (red) corpora; LU, pigs whose ovaries contained more than 6 corpora lutea in absence of follicles larger than 1-2 mm in diameter. 6) The measurement of oxygen and temperature parameters within the lumen of uterus and oviduct and finally, 7) the withdrawal of measure probes and suturing the incision in three planes. The time spent between the beginning of the surgery (skin incision) until the externalization of the reproductive organs was around 5-22 min.

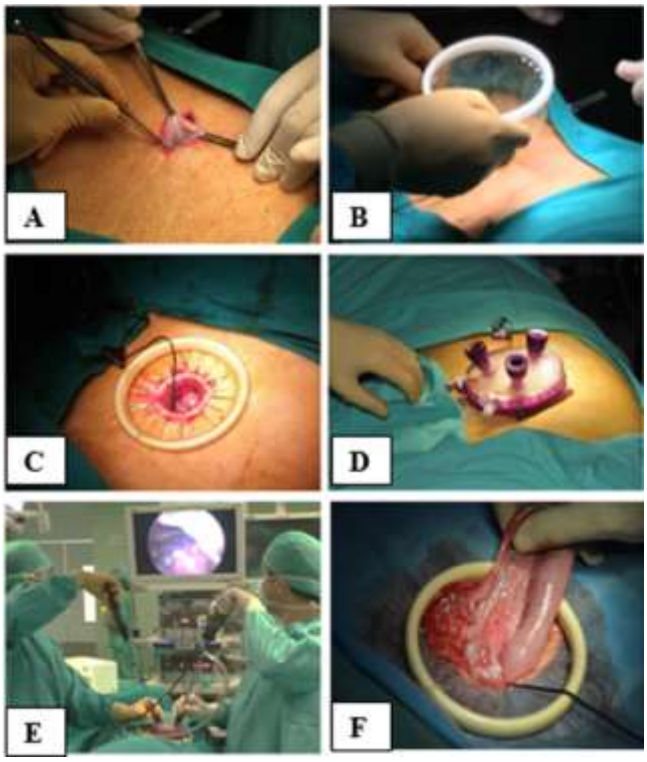

Figure 13. Sequence of the surgical process. A: Incision. B-D: Monoport setup. E: Visualization of internal organs. F: Handling of internal organs.

3.3.5. Measurements of oxygen and temperature levels

The protocol carried out for the collection of oxygen and temperature measurements within different regions of the reproductive tract (ampulla, isthmus and uterus) consisted of the following steps:

1) Implantation of the probe within the organ lumen: this part of the protocol was regarded as the most delicate. Careful attention was paid to avoid any damage to the blood vessels; when bleeding occurred, measurements were discarded. The identification of the oviduct abdominal opening allowed a smooth progression of the probes within the ampulla (Figure 14A). The tip of the sensors was placed at 6-8 cm from the abdominal opening, and kept in position by compressing the sensor wire against the infundibulum wall with non-traumatic microsurgery clamps. For implantation within the isthmus, a passage through the organ wall was created by puncture with a G20 (28 mm) venocath catheter (Figure 14B). The venocath needle was then removed and the sensors passed

through the catheter sheath until properly placement into the isthmus lumen, at no more than 2 cm from the venocath puncture. A similar procedure as described for the oviduct isthmus was followed for implantation of the sensors within the uterine horn. However, in this case, the venocath catheter was thicker (G14, 51 mm) and the tip of the sensors inserted 6-8 cm depth into the lumen. After implantation within the isthmus or uterus, sensors were firmly kept in its place by anchoring them to the mesosalpinx or mesometrium, respectively, by two absorbable surgical stitches or microsurgery clamps. The necessary time to install the probes at any of the three locations in an animal was around 3-7 min.

2) Returning of the organs to anatomical position and stabilization of abdominal physiological environment. Once reproductive organs were placed to their anatomical position within the abdominal cavity the monoport opening was closed with the monoport cap and 5 minutes later oxygen and temperature were recorded.

3) Recording of oxygen and temperature measurements in real time and *in situ*. For oxygen, the probe consisted in a flexible fiber-optic microsensor 0.5 x 1000 mm, which was connected to a recording unit (Ocean Optics, Largo, Florida, USA) (figure 14C) and a laptop computer. A temperature probe was also connected to the recording unit, which according to the manufacturer's instructions, was placed into the abdominal cavity immediately before measurement for precise on-site calibration of the oxygen sensor, and to allow automatic temperature compensated records of oxygen levels. To evaluate potential interference of the oxygen supplied by the anesthetic equipment with the measured levels of oxygen within the oviduct and uterus, trials were carried out in three pigs. During these trials, the ventilation system was switched off for reasonable time (12-15 min). Despite monitoring progressively decreases blood oxygenation and increases hypercapnia, the recorded levels of oxygen within the reproductive organs remained stable. For temperature, the probe consisted in a 0.5 × 520 mm thermistor bead connected with a 20 μm diamel-coated silver wire to 750 mm long polythene-coated wire. The probe was connected to a thermistor amplifier with a single scale from 34 to 40°C and accurate to 0.04°C and programmed for temperature readings each 12 s (ThermaDataTM Logger TCD; E.T.I. Electronic Temperature Instruments, West Sussex,

UK) (López-Gatius & Hunter, 2017) (figure 14D). For both, oxygen and temperature, data were collected every 10-15 seconds for a time period of 10-12 min.

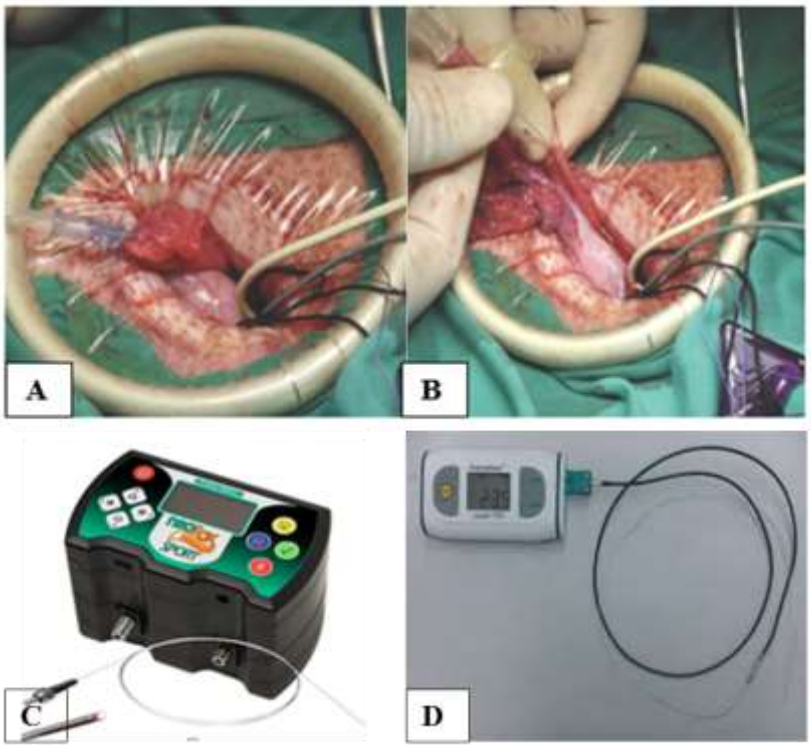

Figure 14. Measurement of O_2 and temperature. A: Implantation of the probe within the oviduct ampulla. B: Implantation of the probe within the oviduct isthmus. C: O_2 luminescent probe and recording unit. D: temperature probe and thermistor amplifier.

3.3.6. Animals recovery

After the surgical procedure, pigs were treated with broad-spectrum antibiotic, amoxicillin/clavulanic acid (20 mg/kg) orally, every 12 hours up to 7 days, and a serial of intramuscular injections of two analgesics, buprenorphine (3 ml single dose) and meloxicam (20 mg/kg), every 12 hours up to 5 days.

3.4. STATISTICAL ANALYSIS

For statistical analysis, IBM SPSS Statistics 22 (Chicago, USA) and PAST 3.02 (free software) were used. The experimental variables of ZP (digestion time), IVF (penetration and monospermy rate, mean number of spermatozoa penetrating per oocyte, percentage of male pronuclear formation and mean number of spermatozoa bound to the zona pellucida) and embryo culture (percentage of cleavage and blastocysts, mean number of cells per blastocyst, embryo development stage and quality) were tested for normality (Shapiro-Wilks test) and homogeneity of variance (Levene test), analyzed by one-way ANOVA and then, data were presented as mean ± standard error of the mean (SEM). The experimental variable of sperm membrane fluidity (calculated diffusion coefficient, CDC) was checked for normal distribution by D'Agostino-Pearson normality test and analyzed by non-parametric Kruskal-Wallis test for equal medians and data were presented as median [25 º percentile- 75 º percentile]. Pearson's correlation coefficient was calculated for temperature and CDC. Kernel Density Estimation (KDE) analysis was applied to identify sub-groups within the sperm population (high fluidity, intermediate fluidity and low fluidity). Descriptive statistics, including mean, SEM, and the 20th, 50th and 80th recorded values of the series were used to characterize each monitored replicate of *in vivo* measurements. Up to three replicates per pig were obtained (ampulla, isthmus and uterus), and potential differences not only due to the sampling site, but also to the sexual maturity of the pigs (gilts *vs.* sows), and the stage of the estrous cycle (early follicular (EF), peri-ovulatory (PO) and luteal (LU)) were evaluated by Levene test and subsequently by double ANOVA (one-way with the means, and repeated measures with the 20th, 50th and 80th recorded data). When ANOVAs revealed a significant effect, values were compared by the post-hoc Tukey test. A p- value<0.05 was considered statistically significant.

3.5. EXPERIMENTAL DESIGN

In this PhD thesis, four experiments were performed to characterize the pig oviductal and uterine environment, studying a few of the factors involved such as proteins previously identified in oviductal secretions, reproductive fluids, oxygen tension, and

temperature, with the aim of transferring this knowledge into the *in vitro* conditions and thus enhance the IVEP systems in swine.

3.5.1. Experiment 1. Effect of previously identified oviductal proteins on different parameters related to porcine *in vitro* fertilization

The purpose of this experiment was to continue with the study carried out by Mondéjar et al. (Mondéjar et al., 2013) about the role of exogenous proteins such as HSPs and ezrin in the oviductal secretions. These proteins, along with OVGP1, were found in abundance within the hardener fraction of the porcine oviductal periovulatory fluid (Mondéjar et al., 2013). Therefore, it was studied whether the addition of HSPs and ezrin to the culture medium, either alone or in combination with the stabilizing glycosaminoglycan heparin, induces a ZP resistance to protease digestion in *in vitro* matured porcine oocytes and, as consequence, improves the IVF parameters. According to figure 2, this experiment was subdivided in two parts.

3.5.1.1. Experiment 1.1. Effect of exogenous proteins and heparin on zona pellucida resistance to protease digestion

Porcine COCs were matured *in vitro* in groups of 10 to 12 and incubated in TALP medium supplemented according to table 10 during 1 h. Three replicates were performed. The ZP digestion time was determined for each group and compared between them. HSP-70-1A (ADI-ESP-555, Enzo Life Sciences, Farmingdale, NY, USA) concentration was set at 530 ng/mL based on its concentration in amniotic fluid of women at term (Chaiworapongsa et al., 2008) and HSP-90α (ADI-SPP-776, Enzo Life Sciences) at 550 ng/mL based on human plasma levels studies (Shi et al., 2014). Based on its characterization, ezrin (ab91744, Abcam, Cambridge, UK) was used at 200 ng/mL (X. Yao, Cheng, & Forte, 1996). Finally, heparin sodium salt (H3393) concentration was set at 1.75 UI/ml (Coy 2008b).

Table 10. Experimental groups in experiment 1.1. Oocyte incubation was done in all groups at 38.5ºC, under 5 % CO_2 and 20 % O_2.

Group	Incubation medium
Control	TALP medium
HSP-701A	TALP medium supplemented with HSP-70-1A
HSP-90α	TALP medium supplemented with HSP-90α
Ezrin	TALP medium supplemented with ezrin
Heparin	TALP medium supplemented with heparin
Heparin + HSP-701A	TALP medium supplemented with heparin and HSP-70-1A
Heparin + HSP-90α	TALP medium supplemented with heparin and HSP-90α
Heparin + ezrin	TALP medium supplemented with heparin and ezrin

3.5.1.2. Experiment 1.2. Effect of proteins and heparin on in vitro fertilization outcome

To test whether proteins with higher ZP digestion times (ZPdt) are correlated with higher monospermy rates, porcine COCs were matured *in vitro*. Next, IVF was performed supplementing the TALP medium with heparin or proteins (HSP-70-1A, HSP-90α and ezrin) at the same concentration described previously. For this experiment, spermatozoa were selected by density gradient centrifugation using Percoll®. Each group consisted of 50 oocytes. Four replicates were performed and a control group of oocytes and sperm co-incubated under the same conditions in TALP medium without proteins or heparin was used in all the replicates (table 11). At 18-20 hpi, putative zygotes were fixed, stained, and examined under epifluorescence microscopy to assess the fertilization rates. IVF parameters were compared between experimental groups and their control.

Table 11. Experimental groups in experiment 2.2. Gametes co-incubation was done in all groups at 38.5ºC, under 5 % CO_2 and 20% O_2.

Group	Gametes co-incubation medium
Control	TALP medium
HSP-70-1A	TALP medium supplemented with HSP-70-1A
HSP-90α	TALP medium supplemented with HSP-90α
Ezrin	TALP medium supplemented with ezrin
Heparin	TALP medium supplemented with heparin

3.5.2. Experiment 2. The impact of simulating the female genital reproductive environment on the outcome of the *in vitro* production of porcine embryos

The purpose of this experiment was to demonstrate whether the use of a more natural sperm selection method in combination with the sequential addition of porcine reproductive fluids during IVF and EC, from the pig oestrous cycle phase that best mimic those found *in vivo*, improves the efficiency of IVEP in swine. According to figure 2, this experiment was subdivided in two parts.

3.5.2.1. Experiment 2.1. The effect of using a more physiological sperm selection method on in vitro fertilization

A swim-up protocol, without centrifugation, where spermatozoa have to swim through a medium imitating, as far as possible, *in vivo* conditions (NaturARTs® PIG sperm swim-up medium, EmbryoCloud) was set up, and compared with a conventional selection system by density gradient centrifugation. In addition, the swim-up medium was supplemented either with BSA (swim-up BSA group) or porcine oviductal periovulatory fluid (swim-up fluid group). To this end, groups of 50-55 porcine COCs were matured *in vitro*. Then, spermatozoa were selected, on the one hand, traditionally in density gradient centrifugation and, on the other hand, by swim-up supplemented either with 1 % NaturARTs® POF-LF or with 0.5 % BSA (table 12). Next, gametes were co-cultured in TALP medium either with or without 1 % NaturARTs® POF-LF from 0-18 hpi (table12).

Presumptive zygotes were fixed, stained, and examined under epifluorescence microscopy to assess the fertilization rates.

Table 12. Experimental groups in experiment 2.1. Gametes co-incubation was done in all groups at 38.5ºC, under 5 % CO_2 and 20% O_2.

Group	Sperm preparation	IVF
Percoll®	Density gradients centrifugation	TALP
Swim-up BSA	Swim-up + BSA	TALP
Swim-up fluid	Swim-up + POF-LF	TALP + POF-LF

3.5.2.2. Experiment 2.2. Effect of oviductal and uterine fluid used as additives in the culture media on in vitro fertilization and further embryo culture

Groups of 50-55 porcine COCs were matured *in vitro*. Then, spermatozoa were selected by swim-up supplemented either with 1 % NaturARTs® POF-LF or with 0.5 % BSA (table 13). Next, gametes were co-cultured in TALP medium either with or without 1 % NaturARTs® POF-LF from 0-18 hpi (table 13). Then, putative zygotes were cultured until blastocyst stage in NCSU-23 medium either with or without fluids (table 13). NCSU-23A with or without 1 % NaturARTs® POF-EL was used from 18 to 48 hpi of embryo culture and NCSU-23B with or without 1 % NaturARTs® PUF-LL was used from 48-180 hpi. At 48 hpi, cleavage was evaluated under the stereomicroscope. Finally, at 180 hpi, development to the blastocyst stage was assessed finishing with fixing and staining to count the mean cell number per blastocyst. Five replicates were performed.

Table 13. Experimental groups in experiment 2.2. IVF and EC were done in all groups at 38.5ºC, under 5 % CO_2 and 20% O_2.

Group	Sperm preparation	IVF	EC 0-2days	EC 3-7days
Control	Swim-up + BSA	TALP	NCSU-23A	NCSU-23B
Fluids	Swim-up + POF-LF	TALP + POF-LF	NCSU-23A + POF-EL	NCSU-23B + PUF-LL

3.5.3. Experiment 3. Effect of physiological oxygen tension on the outcome of the *in vitro* production of pig embryos

The purpose of this experiment was to determinate the oxygen tension within the oviduct and uterus of the female pig during the different phases of the oestrus cycle in order to demonstrate whether the use of an oxygen atmosphere closer to those found physiologically within the genital tract might improve the efficiency of IVEP in swine. According to figure 2, this experiment was subdivided in two parts.

3.5.3.1. Experiment 3.1. In vivo measurement of oxygen tension within the reproductive tract of female pigs

Records of oxygen tension were performed *in situ* in 29 pigs, following a LESS assisted approach. Data were then grouped and compared according to the pig age (16 gilts and 13 sows), phase of the oestrous cycle (8 EF, 15 PO and 6 LU) and the site where the sensor was implanted (26 ampulla, 25 isthmus and 13 uterus). Then, data were analyzed, independently for gilts and sows. From the 16 gilts: 7 EF and 9 PO, the sensor was implanted in the ampulla of the 16 gilts, the isthmus of 12 gilts and the uterus of 8 gilts. From the 13 sows: 2 EF, 5 PO and 6 LU, the sensor was implanted within the ampulla of 9 sows, the isthmus of 8 sows and the uterus of 5 sows.

3.5.3.2. Experiment 3.2. Effect of physiological oxygen tension on the outcome of the in vitro production of porcine embryos

After IVM (groups of 50 to 55 COCs), IVF followed by EC were performed at two different oxygen tensions: those widely used for the pig reproduction centers, atmospheric oxygen (20 %) or the mean *in vivo* value obtained from sows in experiment 3.1. (7 %) in an incubator containing 88 % N_2 (Labotec C60, Göttingen, Germany). Experimental conditions are summarized in table 14. In addition, for both groups, IVF medium (TALP) was supplemented with 1 % NaturARTs® POF-LF and the EC media (NCSU-23A and 23B) were supplemented with 1 % NaturARTs® POF-EL for the first 48 hpi and then, with 1 % NaturARTs® PUF-LL up to 180 h, respectively. At 18 hpi, a small sample of presumptive zygotes were fixed, stained, and examined under epifluorescence microscopy to assess the fertilization rates. At 48 hpi, cleavage was

evaluated under the stereomicroscope. Finally, at 180 hpi, development to the blastocyst stage was quantified and blastocysts were fixed and stained to count the mean cell number per blastocyst. Five replicates were performed.

Table 14. Experimental groups in experiment 3.2. IVM, IVF and EC were done in all groups at 38.5ºC, under 5 % CO_2.

Group	IVM	IVF	EC 0-2days	EC 3-7days
20 % O_2	20 % O_2	20 % O_2	20 % O_2	20 % O_2
7 % O_2	20 % O_2	7 % O_2	7 % O_2	7 % O_2

3.5.4. Experiment 4. Effect of physiological temperature on the outcome of the *in vitro* production of porcine embryos

The purpose of this experiment was to determinate the temperature within the oviduct and uterus of the female pig during the different phases of the oestrus cycle in order to demonstrate whether the use of a temperature closer to those found physiologically might improve the efficiency of IVEP in swine. According to figure 2, this experiment was subdivided in three parts.

3.5.4.1. Experiment 4.1. In vivo measurement of temperature within the reproductive tract of female pigs

Continuous readings of temperature were performed in 31 pigs, following LESS assisted approach. Readings were then grouped and compared according to the pig age (16 gilts and 15 sows), pig's phase of the oestrous cycle (6 EF, 21 PO and 7 LU) and the site where the sensor was implanted (18 ampulla, 21 isthmus and 17 uterus). Then, data were analyzed, independently for gilts and sows. From the 16 gilts: 4 EF and 12 PO, the sensor was implanted in the ampulla of 12 gilts, in the isthmus of 15 gilts and in the uterus of 6 gilts. From the 15 sows: 2 EF, 6 PO and 7 LU, the sensor was implanted within the ampulla and isthmus of 6 sows and within the uterus of 11 sows.

3.5.4.2. Experiment 4.2. Effect of temperature on sperm structure and functionality

To study the effect of the different physiological temperature found in experiment 4.1 (37.0, 38.5 and 39.5 ºC) on sperm functionality that might lead to modification of the

IVEP outcomes, structural changes in the sperm membrane were analyzed. Therefore, firstly, it was determined the temperature transition phase of lipid in the membrane of boar sperm by differential scanning calorimetry (DSC). The temperature transition phase of lipid is defined as the temperature required to induce a structural change in the lipid physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid. Secondly, boar spermatozoa were incubated at 37.0, 38.5 and 39.5 ºC and sperm membrane fluidity was assessed by fluorescence recovery after photobleaching (FRAP). Three independent replicates were performed using different boars and on different days for each procedure.

3.5.4.3. Experiment 4.3. Effect of different temperatures on the outcome of the in vitro production of porcine embryos

IVM, IVF and EC were performed in a cube drawer type incubator (ASTEC (AD-3100), IVFSynergy, Truro, United Kingdom) using Nidoil (Nidacon, Barcelona, Spain) during IVF and EC. IVF was performed at three different temperatures: 37.0, 38.5 and 39.5 ºC. Experimental conditions are summarized in table 15. At 18-20 hpi, a small sample of presumptive zygotes were fixed, stained, and examined under epifluorescence microscopy to assess the fertilization rates. At 48 hpi, cleavage was evaluated under the stereomicroscope. Finally, at 180 hpi, development to the blastocyst stage was quantified, blastocyst morphology was assessed, and blastocysts were fixed and stained to count the mean cell number per blastocyst. Five replicates were performed.

Table 15. Experimental groups in experiment 4.3. IVM, IVF and EC were performed under 5 % CO_2 and hypoxia conditions (7 % O_2). Temperature was set at 38.5 ºC during IVM and EC.

Group	IVM	IVF	EC 0-2days	EC 3-7days
37.0ºC	38.5 ºC	37.0 ºC	38.5 ºC	38.5 ºC
38.5 ºC	38.5 ºC	38.5 ºC	38.5 ºC	38.5 ºC
39.5 ºC	38.5 ºC	39.5 ºC	38.5 ºC	38.5 ºC

4. RESULTS

4.1. EXPERIMENT 1

4.1.1. Experiment 1.1. Incubation of pig oocytes with exogenous oviductal proteins and heparin slightly hardens the ZP

As it is shown in figure 15, the time of ZP solubility increased when *in vitro* matured porcine oocytes were incubated indistinctly in presence of heparin or proteins. This hardening effect was enhanced when the oocytes were incubated in presence of both heparin and proteins, except for HSP-90α. The ZP digestion time (ZPdt) for oocytes incubated with the different proteins varied depending on the protein used. Oocytes incubated in a medium that contained ezrin showed the higher effect of hardening (113.4 ± 3.3 s, n=31) and this time increased when heparin was added to the culture medium (177.1 ± 17.2 s, n=28). Proteins belonging to the same family, such as Heat Shock Proteins HSP-70-1A and HSP-90α, had a similar ZPdt (81.2 ± 2.8 s, n=31) and (79.7 ± 3.3 s, n=31), respectively, and those times were similar to heparin group digestion time that was around 80 s (n=121). However, when heparin was also added to the culture media, the ZPdt increased for HSP-70-1A (103.6 ± 7.5 s, n=30) whereas decreased for HSP-90α (66.6 ± 2.7 s, n=31) to control level, that was around 60 s (n=136).

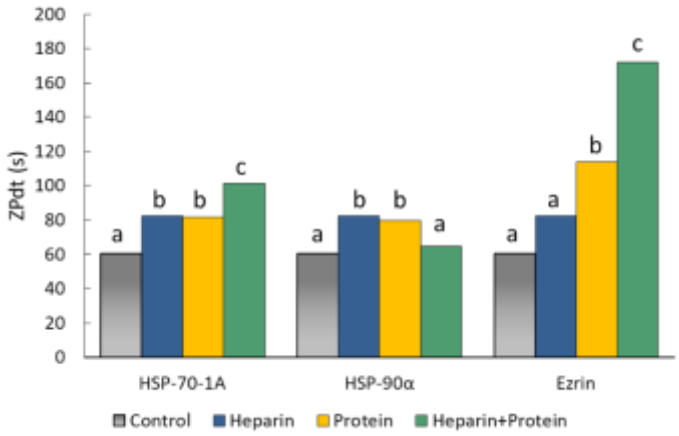

Figure 15. ZP digestion time of *in vitro* matured porcine oocytes after incubation without (control) or with different proteins or/and heparin. Different letters within the same protein were used to indicate significantly different values (P<0.05).

4.1.2. Experiment 1.2. IVF outcome is not improved by addition of exogenous oviductal proteins and heparin to gamete co-incubation medium

Table 16 shows the results of this experiment. Culture of gametes within an IVF culture medium supplemented with heparin or any of the proteins decreased, significantly, penetration rates and the mean number of sperm penetrated per oocyte while increased the monospermy rates compared to the control group. Moreover, these differences were more remarkable in ezrin and heparin groups, where the number of sperm bound to the zona pellucida also decreased significantly. Male pronuclear formation % (MPN) and IVF yield were no affected by the addition of heparin or proteins to the IVF medium.

Taking into consideration the results obtained in this experiment, and since no improvements in the IVF yield were observed, the direct addition of porcine reproductive fluids to the culture media in the second experiment was studied.

Table 16. IVF data (mean ± SEM) for the addition of heparin, HSP-70-1A, HSP-90α or ezrin to the IVF culture medium. Heparin and each protein was compared separately to a control group.

Group	N	Penetration (%)	Monospermy (%)	S/O	MPN (%)	ZPb	IVF yield (%)
Control	114	87.7 ± 3.1 a	20.4 ± 3.7 a	3.6 ± 0.3 a	99.0 ± 1.0	47.5 ± 3.8 a	17.5 ± 3.6
Heparin	107	30.8 ± 4.5 b	84.8 ± 4.2 b	1.1 ± 0.1 b	100	29.6 ± 3.2 b	26.2 ± 4.3
Control	177	71.7 ± 3.4 a	20.6 ± 3.6 a	3.2 ± 0.2 a	97.6 ± 1.4	28.9 ± 1.1	14.7 ± 2.7
HSP-70-1A	171	54.4 ± 3.8 b	40.9 ± 5.1 b	2.2 ± 0.1 b	100	29.6 ± 1.3	22.2 ± 3.2
Control	176	71.6 ± 3.4 a	22.2 ± 3.7	3.2 ± 0.2 a	97.6 ± 1.4	28.4 ± 1.2	15.9 ± 2.8
HSP-90α	161	60.9 ± 3.9 b	33.7 ± 4.8	2.2 ± 0.1 b	100	30.2 ± 1.1	20.5 ± 3.2
Control	173	71.1 ± 3.5 a	21.3 ± 3.7 a	3.2 ± 0.2 a	97.5 ± 1.4	28.7 ± 1.2 a	15.0 ± 2.7
Ezrin	191	28.3 ± 3.3 b	74.1 ± 6.0 b	1.4 ± 0.1 b	100	20.9 ± 1.7 b	20.9 ± 2.9

N: number of matured oocytes. In columns, a, b: different letters indicate values statistically different ($P<0.05$)

4.2. EXPERIMENT 2

4.2.1. Experiment 2.1. Sperm selection using a more physiological method improves IVF outcomes

This experiment was designed to select sperm using a more natural way before IVF. The results are shown in table 17. Evaluation at 18-20 hpi, revealed that the efficiency of IVF improved by using swim-up as sperm selection method obtaining significantly higher rates of monospermy than density gradient centrifugation method and a higher percentage of putative zygotes. Besides, the addition of porcine oviductal periovulatory fluid to the swim-up medium instead of BSA increased the final yield of the system. When swim-up groups were compared, swim-up fluid showed similar penetration rate but higher monospermy rates than swim-up BSA group.

Table 17. IVF parameters data (mean ± SEM) at 18-20 hpi of porcine oocytes inseminated by using three different sperm selection protocols.

Group	N	Penetration (%)	Monospermy (%)	S/O	ZPb	IVF yield (%)
Density gradient centrifugation	105	84.3 ± 3.6 a	17.4 ± 4.1 a	8.4 ± 0.7 a	17.3 ± 2.3 a	14.6 ± 0.1 a
Swim-up BSA	180	69.6 ± 3.5 b	42.7 ± 4.6 ab	2.1 ± 0.1 b	7.2 ± 0.5 b	29.7 ± 0.2 b
Swim-up fluid	183	71.1 ± 3.4 b	49.6 ± 4.5 b	2.7 ± 0.1 b	8.6 ± 0.5 b	35.2 ± 0.2 c

N: number of inseminated oocytes. In columns, a, b, c: different letters indicate values statistically different ($P<0.05$)

4.2.2. Experiment 2.2. Reproductive fluids used as additives within the culture media improves the IVEP outcomes

This experiment was designed to select the best culture media for the IVEP. Regarding embryo development, more than 40 % of cleaved embryos reached the blastocyst stage in both groups (fluids and control) at 48 hpi (table 18). Evaluation at 180 hpi revealed that the addition of reproductive fluids to the fertilization and embryo culture media increased blastocyst quality, since fluids group showed a higher mean cell number per blastocyst (81.8 ± 7.2) compared with the same system without fluids (49.9 ± 3.6),

and that number was similar to *in vivo* collected blastocysts (87.0 ± 7.2) (table 18). Moreover, fluids group showed a significant increase in the proportion of hatching and hatched blastocysts, compared with the control group where no hatching was observed at day 7.5 (table 18).

Table 18. Pig embryo development data at 48 and 180 hpi. Porcine oocytes were inseminated with sperm selected by swim-up method and cultured either with (fluids) or without (control) porcine reproductive fluids added to the culture media.

Group	N	Cleavage, n (mean ± SEM) (%)	Blastocysts, n (mean ± SEM) (%) (over total of cleaved)	Blastocysts (mean ± SEM) (%) (over total of putative zygotes)	Cell/blastocyst (mean ± SEM) (%)
Control	903	429 (47.5 ± 1.6) a	178 (41.4 ± 2.4)	19.6 ± 1.3	49.9 ± 3.7 a
Fluids	961	405 (42.1 ± 1.6) b	180 (44.5 ± 2.5)	18.7 ± 1.2	81.8 ± 7.2 b
In vivo	41	N/A	N/A	N/A	87.0 ± 7.2 b

Group	Early Blastocyst, n (mean ± SEM) (%)	Blastocyst, n (mean ± SEM) (%)	Expanded blastocyst, n (mean ± SEM) (%)	Hatching blastocyst, n (mean ± SEM) (%)	Hatched blastocyst, n (mean ± SEM) (%)
Control	57 (31.7 ± 6.1) a	50 (28.3 ± 5.9)	71 (40.0 ± 6.4)	0 a	0 a
Fluids	23 (12.8 ± 5.4) b	55 (30.8 ± 7.5)	65 (35.9 ± 7.8)	28 (15.4 ± 5.9) b	9 (5.1 ± 3.6) b

N: No. of putative zygotes. In columns, a, b: different letters indicate values statistically different ($P<0.05$)

4.3. EXPERIMENT 3

4.3.1. Experiment 3.1. Minimally invasive procedures are able to obtain oxygen measurement within the reproductive tract of female pigs with success

After implantation of the probe within the organ and the stabilization period, the display of the monitored oxygen tension in each record showed two different profiles (figure 16). One profile was a flat line with minimum variation from the average, and the other profile showed a waved pattern with peaks as low or high as 1.97-2.63 % from the mean, and wave lengths as long as 0.5-3 min. A profile was considered as waved when at least two waves deviating more than 1.3 % oxygen from the mean or 1 wave longer than 2 min were observed. Both profiles were shown in the two groups of pigs (gilts and

sows), the three stages of the oestrous cycle (EF: early follicular; PO: peri-ovulatory; LU: luteal) and the three measuring sites (ampulla, isthmus and uterus) for gilts but only in ampulla and isthmus for sows. Chi-squared test showed a significant negative association of the flat profile with the uterus ($P< 0.05$). Regarding the results of the tension within the reproductive organs, the average oxygen tension percentage was always substantially lower (8.5 % O_2) compared to ambient air (20 % oxygen). Oxygen readings ranged from 6.0 to 11.0 %. A significant difference in oxygen tension was observed depending on the animal age being higher in gilts (10.1 ± 1.6 %) than sows (7.6 ± 1.8) ($P<0.05$) (figure 17). This difference was consistently found in the oviduct ampulla (10.8 ± 1.7 vs. 7.0 ± 1.0, $P<0.05$) and isthmus (9.6 ± 1.5 vs. 6.5 ± 1.0, $P<0.05$) for gilts and sows, respectively. However, the oxygen percentage in the uterus was similar between gilts and sows (9.2 ± 1.1 vs. 10.3 ± 1.5). When the results were analysed separately for gilts and sows, additional differences were observed. In gilts, the oxygen tension in the uterus was significantly lower than in the ampulla, while in sows it was significantly higher than in the isthmus (table 19). Finally, no significant changes in the oxygen tension were observed throughout the estrous cycle in both gilts and sows, although a trend towards higher oxygen tension at the peri-ovulatory stage (PO) was noticed (figure 17 and table 19).

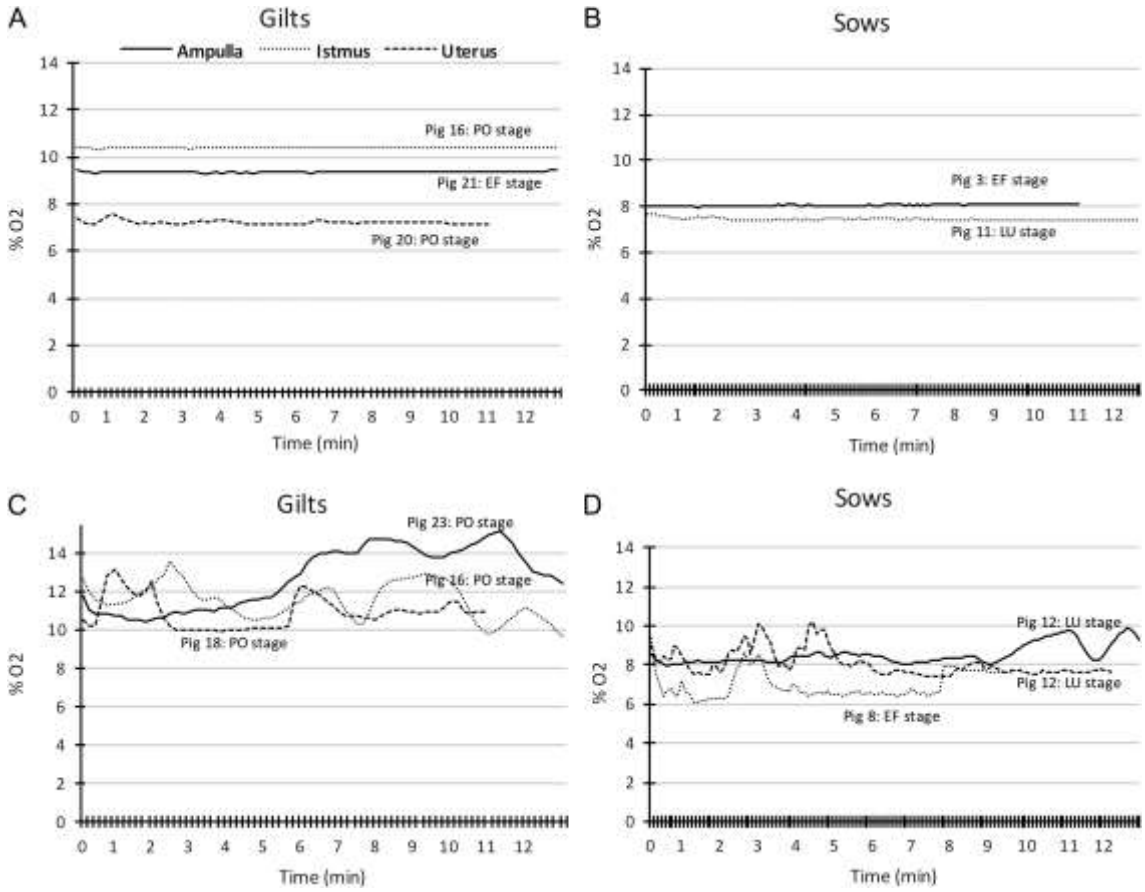

Figure 16. Flat and waved profiles displayed during oxygen records in the reproductive tract of female pigs. A and C: A few samples of the oxygen profiles of different reproductive organs in gilts at different estrous cycle stages. B and D: A few samples of the oxygen profiles of different reproductive organs in sows at different estrous cycle stages. None of the sows showed a flat oxygen profile in the uterus (B).

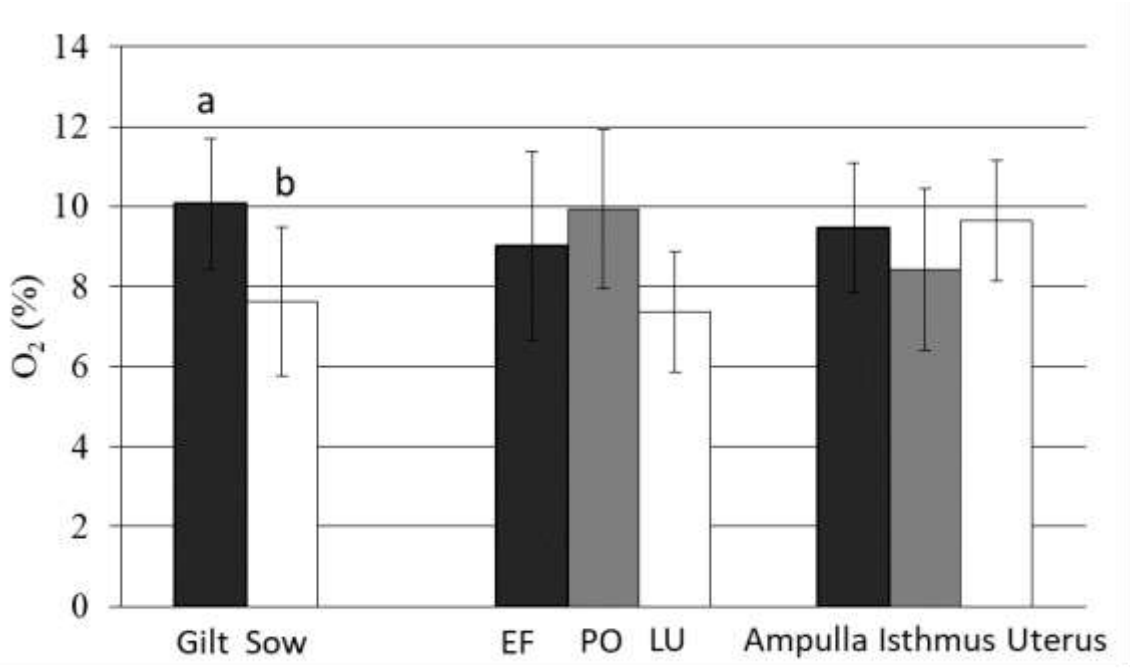

Figure 17. Percentage of oxygen in the female reproductive tract related to the age of the pigs (gilts and sows), the estrous cycle (early follicular-EF, periovulatory-PO and late luteal-LU); and the sampling sites (ampulla, isthmus and uterus). Gilts and sows data were combined in the last two groups of bars of the graphic. Different letters were used to indicate significantly different values (P<0.05).

Table 19. Oxygen tension (mean ± SEM) for gilts and sows related to the sampling site (ampulla, isthmus and uterus) and the stage of the estrous cycle (early follicular-EF, periovulatory-PO and luteal-LU). Different letters were used to indicate significantly different values within gilts and sows groups.

	SAMPLING SITE			**STAGE OF THE ESTROUS CYCLE**		
	Ampulla	**Isthmus**	**Uterus**	**EF**	**PO**	**LU**
Gilts	10.8 ± 1.7 a	9.6 ± 1.5 ab	9.2 ± 1.1 b	9.7 ± 1.1	10.2 ± 1.5	N/A
Sows	7.0 ± 1.0 ab	6.5 ± 1.0 a	10.3 ± 1.5 b	6.9 ± 0.8	8.6 ± 1.0	7.4 ± 1.0

In the same row, a, b: different letters indicate values statistically different (P<0.05)

4.3.2. Experiment 3.2. The use of an oxygen tension similar to those measured within the reproductive tract of female pigs during IVF and EC enhances the efficiency of porcine IVEP and the quality of the blastocysts obtained

The efficiency of IVF was assessed under two different oxygen conditions (20 and 7 % oxygen in air). Twenty percent was used as control because this is the standard oxygen concentration used in most pig reproduction laboratories. Seven percent was selected as reference value from the experiment 3.1 because represented the natural conditions in which fertilization and embryo development take place in *in vivo* conditions, where adult animals are used for reproductive purposes.

The two oxygen tensions along with the addition of reproductive fluids to the IVF and EC media were suitable for performing with success the ART. Furthermore, physiological versus ambient oxygen concentration had no effect on *in vitro* fertilization parameters (table 20); however, preimplantation embryo development was affected by low oxygen conditions (table 21). Cleavage rate of porcine *in vitro* fertilized embryos maintained under 7 % oxygen during IVF and EC was significantly higher compared with those cultured under 20 % oxygen, remarkably improving the efficiency of the culture system (blastocysts % over total of putative zygotes). In addition, evaluation of embryo development and quality revealed that IVF and EC under low oxygen concentrations increased blastocyst quality since blastocysts cultured under 7 % oxygen had increased mean number of cells compared to those cultured under 20 % oxygen. Although no significant differences were appreciated for different embryo developmental stages between the groups due to the limited number of blastocysts (25 from the 20% oxygen, and 50 from the 7% oxygen groups), the absolute values for hatching and hatched blastocysts were larger in embryos cultured under 7% oxygen than under 20%.

Table 20. IVF data (mean ± SEM) for porcine oocytes inseminated under 20 or 7 % oxygen assessed at during18-20 hours. N: No. of matured oocytes.

O_2 (%)	N	Penetration (%)	Monospermy (%)	S/O	ZPb	IVF Yield (%)
20	271	55.0 ± 3.0	64.0 ± 3.9	1.5 ± 0.1	8.2 ± 0.4	36.0 ± 2.9
7	238	60.0 ± 3.2	59.0 ± 4.1	1.7 ± 0.1	9.1 ± 0.5	36.0 ± 3.1

Table 21. Pig embryo development data at 48 and 180 hpi for two oxygen concentrations (20 % and 7 %) during IVF and IVC.

Group	**N**	**Cleavage, n (mean ± SEM) (%)**	**Blastocysts, n (mean ± SEM) (%) (over total of cleaved)**	**Blastocysts (mean ± SEM) (%) (over total of putative zygotes)**	**Cell/blastocyst (mean ± SEM) (%)**
20 % O_2	471	153 (32.0 ± 2.2) a	25 (16.0 ± 3.0)	5.0 ± 1.0 a	59.0 ± 5.0 a
7 % O_2	467	281 (60.0 ± 2.3) b	50 (18.0 ± 2.3)	11.0 ± 1.4 b	88.9 ± 5.9 b

Group	**Early Blastocyst, n (mean ± SEM) (%)**	**Blastocyst, n (mean ± SEM) (%)**	**Expanded blastocyst, n (mean ± SEM) (%)**	**Hatching blastocyst, n (mean ± SEM) (%)**	**Hatched blastocyst, n (mean ± SEM) (%)**
20 % O_2	0	6 (24.0 ± 8.7)	13 (52.0 ± 10.2)	3 (12.0 ± 6.6)	3 (12.0 ± 6.6)
7 % O_2	1 (2.0 ± 2.0)	11 (22.0 ± 5.9)	17 (34.0 ± 6.8)	11 (22.0 ± 5.9)	10 (20.0 ± 5.7)

N: No. of putative zygotes. a, b: different letters in columns indicate values statistically different ($P<0.05$)

4.4. EXPERIMENT 4

4.4.1. Experiment 4.1. Minimally invasive procedures are able to obtain temperature measurement within the reproductive tract of female pigs with success

Regarding the results of temperature within the reproductive organs, the average temperature was 38.0 ºC, but a marked individual variability among animals was observed. The average values varied from 35.8 to 41.3 ºC. A significant difference in temperature was found between ampulla (37.5 ± 0.3) and uterus (38.5 ± 0.1) (figure 18). No differences were found depending on the animal age and the phase of the oestrous cycle (figure 18). Nevertheless, when the results were analysed separately for gilts and

sows, additional information was found. While in gilts, no differences were observed, in sows the same temperature was found in ampulla and isthmus (37.0 ± 0.5) and this one was significantly lower compared with that found in uterus (38.7 ± 0.1) (table 22). Finally, no significant changes in the temperature were observed throughout the estrous cycle in both gilts and sows, although a trend towards higher temperature at the peri-ovulatory (PO) and luteal (LU) stages was observed in sows (table 22).

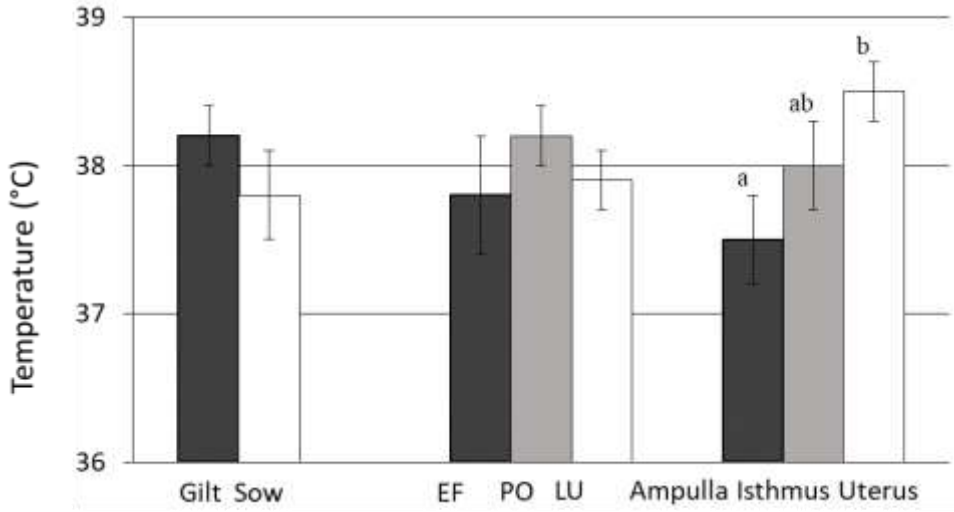

Figure 18. Temperature within the female reproductive tract related to the sexual maturity of the pigs (gilts and sows), the stage of the estrous cycle (early follicular-EF, periovulatory-PO and late luteal-LU) and the sampling sites (ampulla, isthmus and uterus), where gilts and sow's data were combined. Different letters were used to indicate significantly different values (P<0.05).

Table 22. Temperature (mean ± SEM), separately, in gilts and sows related to the sampling site (ampulla, isthmus and uterus) and the stage of the estrous cycle (early follicular-EF, periovulatory-PO and luteal-LU). Different letters were used to indicate significantly different values within gilts and sows' groups.

	SAMPLING SITE			**STAGE OF THE ESTROUS CYCLE**		
	Ampulla	**Isthmus**	**Uterus**	**EF**	**PO**	**LU**
Gilts	37.8 ± 0.3	38.4 ± 0.4	38.4 ± 0.3	38.4 ± 0.4	38.1 ± 0.3	N/A
Sows	37.0 ± 0.5 a	37.0 ± 0.5 a	38.7 ± 0.1b	36.7 ± 0.3	38.2 ± 0.3	37.9 ± 0.5

a, b: different letters in the same row and sampling site indicate values statistically different (P<0.05)

4.4.2. Experiment 4.2. Membrane sperm composition is reorganized during its exposition to minimal temperature changes

The temperature transition phase for the boar sperm membrane was initiated at 35.5 ºC and ended at 40.9 ºC, reaching its peak at 37.1 ºC (figure 19). This last one could be then considered as the transition temperature for capacitated boar sperm membrane phase behaviour, where there is a balance in the physical status of the membrane lipids. Finally, the enthalpy of the process was -1.1 J/g (figure 19).

The Analysis of FRAP revealed an increment in the DilC12 diffusion coefficient from 3.3 [2.2–8.1] (control group, spermatozoa at 0 h) to 6.3 [4.4–12.1] for 37.0 group. Although, 38.5 ºC group showed a diffusion coefficient of 5.4 [2.2–10.1], similar to the 37.0 group, this value was similar statistically to the control group as well (figure 20). When spermatozoa were incubated at 39.5 ºC, this group revealed a CDC of 2.5 [1.4–8.9], similar to the control group (3.3 [2.2–8.1]), and that value was statistically similar to the value of 38.5 ºC group (5.4 [2.2–10.1]) as well (figure 20). When the three temperatures were analysed by Pearson test, it was found a strong negative correlation between the CDC and temperature (r = - 0.96). Finally, when the different sperm sub-populations were studied for each temperature by using Kernel Density estimation (KDE) analysis, new information was obtained. Two different sperm sub-populations were identified in the 37.0 ºC group: low fluidity (CDC values ranging from 3.0 to 12.0) and high fluidity (CDC from 12.0 to 18.0) whereas three sub-populations were identified in the 38.5 and 39.5 ºC groups (low, CDC: 3.0–9.0; intermediate, CDC: 6.0–12.0; and high fluidity, CDC: 12.0–21.0). A graphical illustration of the different sperm sub-populations for each temperature is shown in Figure 21.

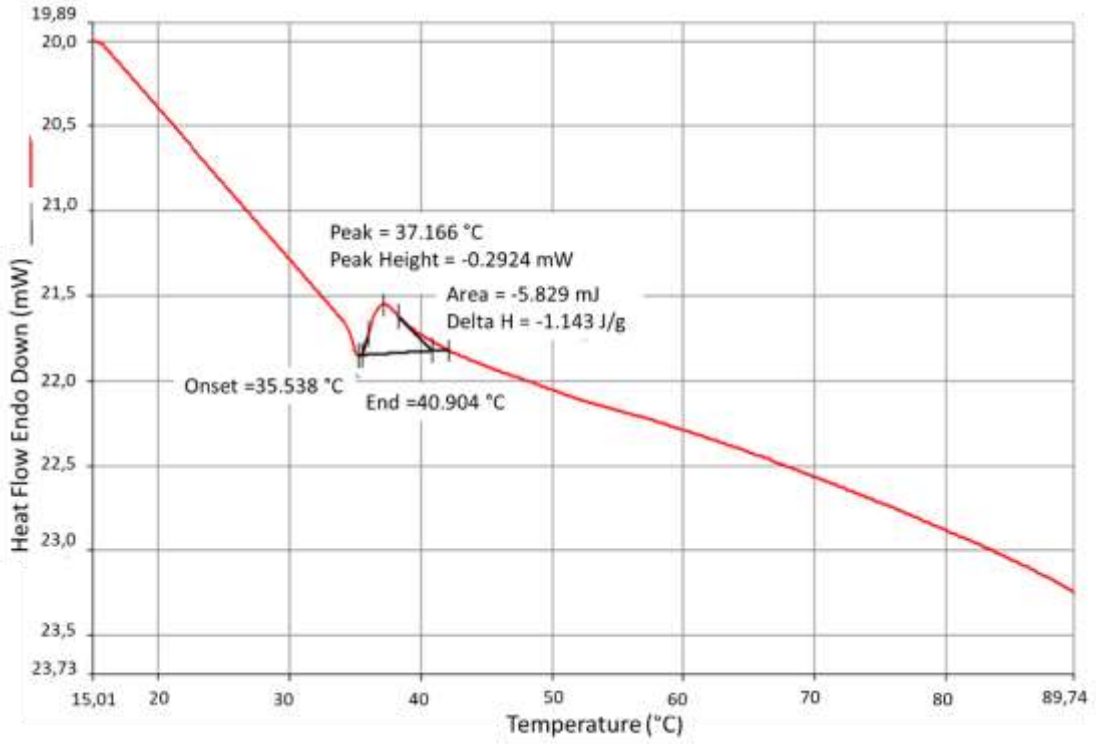

Figure 19. Temperature transition phase for the membrane of capacitated boar sperm.

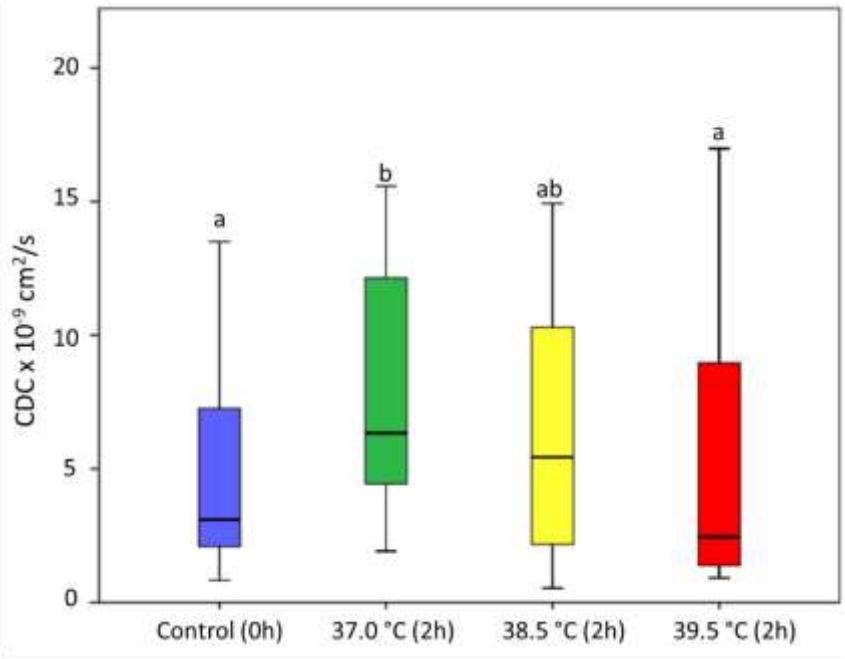

Figure 20. Boxplot (median [25 º percentile – 75 º percentile]) of spermatozoa at time 0 (control) or incubated at different temperatures (37.0, 38.5 and 39.5 ºC) and analysed by FRAP. Different letters were used to indicate significantly different values ($P<0.05$). P values were obtained by comparing the different data sets with Kruskal-Wallis test followed by Mann-Whitney pairwise post-hoc test. A total number of 161 spermatozoa were analysed.

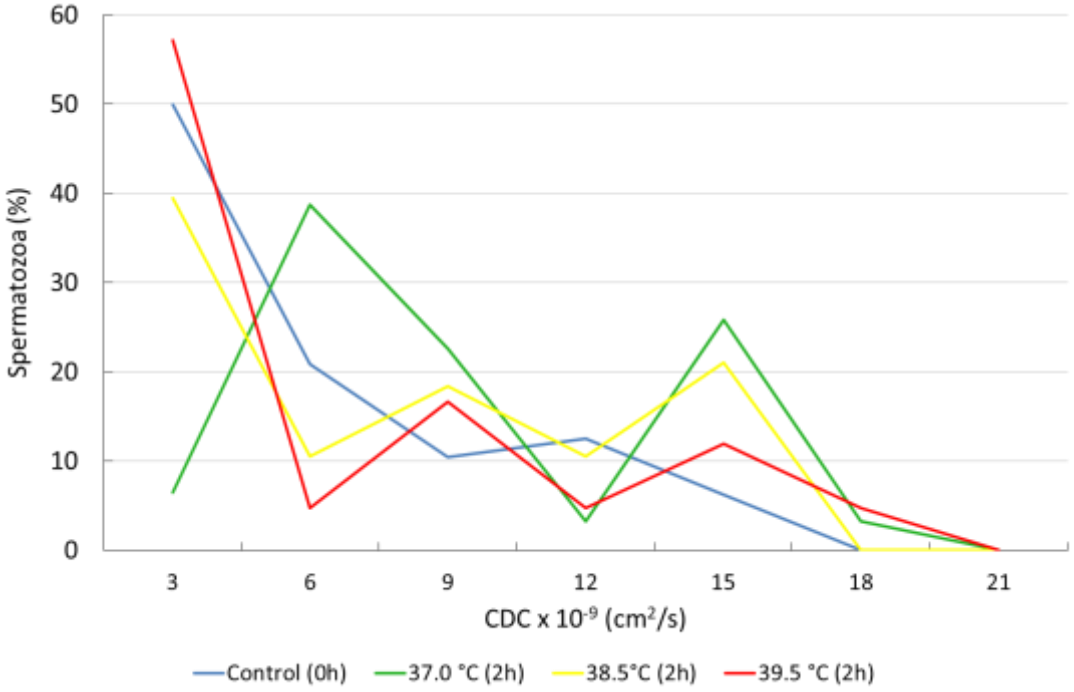

Figure 21. Different sperm sub-populations found by using KDE analysis at different temperatures.

4.4.3. Experiment 4.3. A temperature of 37.0 ºC during IVF enhances the efficiency of porcine IVEP

The efficiency of IVF and EC was assessed using three different temperature conditions (37.0 ºC, 38.5 ºC and 39.5 ºC) during IVF. Low temperature (37.0 ºC) was selected as reference value from the experiment 4.1 because represented the temperature value within the oviduct of adult animals and hence, the natural conditions in which fertilization might take place in *in vivo* conditions. Intermediate temperature (38.5 ºC) was used as a control because is the standard temperature used in pig reproduction laboratories (pig body temperature). High temperature (39.5 ºC) was used to explore the effect of high temperatures during ART since a few of the animals of experiment 4.1 had temperature within the reproductive tract that ranged from 39.0 ºC to 40.0 ºC.

The three temperatures along with hypoxia conditions were suitable for performing with success the ART. The results are shown in tables 23 and 24. Evaluation at 18-20 hpi showed penetration and monospermy rates of 65.0 ± 4.7 and 46.0 ± 6.1, respectively for the control group (38.5 ºC). The use of a high temperature (39.5 ºC)

during IVF increased penetration rates (79.0 ± 3.8) and decreased monospermy rates (31.0 ± 5.0) compared to the use of a low temperature (37.0 ºC), which had similar penetration rates than the control group (54.0 ± 4.7) but higher monospermy rates (65.0 ± 6.1) (table 23). In addition, 37.0 ºC group showed the lowest number of spermatozoa penetrated per oocyte (table 23). Although no statistical differences were observed when IVF yields were compared, the use of 37.0 ºC as culture temperature during IVF showed higher efficiency in absolute values than 38.5 and 39.5 ºC. At 48 hpi, there was observed a significant increase of cleavage rate in the group cultured at 39.5 ºC during IVF (table 24). However, the evaluation after 180 hpi revealed that the group cultured at 37.0 ºC during IVF increased blastocyst yield (over total of embryos) by almost two-fold. Embryo developmental stage, embryo morphological quality and mean number of cells per blastocyst were not affected by the temperature during IVF (table 24).

Table 23. IVF data (mean ± SEM) for porcine oocytes fertilized at different temperature (38.5, 37.0 and 39.5 ºC) after 18-20 hpi. N: number of matured oocytes. A: Penetration and monospermy rates. B: Mean number of sperm penetrated per oocyte (S/O), mean number of sperm bound to the zona pellucida (ZPb) and percentage of putative zygotes obtained (yield). IVM was run at 38.5 ºC. IVM, IVF and EC were run under 7% O_2.

Temperature (ºC)	N	Penetration (%)	Monospermy (%)	S/O	ZPb	IVF yield (%)
37.0	114	54.0 ± 4.7 a	65.0 ± 6.1 a	1.6 ± 0.2 a	7.3 ± 0.8 a	35.0 ± 4.5
38.5	104	65.0 ± 4.7 ab	46.0 ± 6.1 ab	2.4 ± 0.3 b	9.3 ± 1.2 ab	30.0 ± 4.5
39.5	112	79.0 ± 3.8 b	31.0 ± 5.0 b	3.1 ± 0.1 b	11.6 ± 1.9 b	25.0 ± 4.1

N: No. of inseminated oocytes. In columns, a, b: different letters indicate values statistically different ($P<0.05$)

Table 24. Pig embryo development data and morphological quality (mean ± SEM) at 48 and 180 hpi for three temperatures (37.0, 38.5 and 39.5 ºC) during IVF. IVM and EC were run at 38.5 ºC. IVM, IVF and EC were run under 7% O_2.

Group	N	Cleavage, n (mean ± SEM) (%)	Blastocysts, n (mean ± SEM) (%) (over total of cleaved)	Blastocysts (mean ± SEM) (%) (over total of putative zygotes)	Cell/blastocyst (mean ± SEM) (%)
37.0 ºC	374	188 (53.0 ± 2.6) a	73 (39.0 ± 3.6) a	20.0 ± 2.1	50.2 ± 2.5
38.5 ºC	419	236 (56.0 ± 2.4) a	56 (24.0 ± 2.8) b	13.0 ± 1.7	48.1 ± 2.4
39.5 ºC	320	217 (68.0 ± 2.6) b	61 (28.0 ± 3.1) b	19.0 ± 2.2	48.2 ± 2.5

Group	Early Blastocyst, n (mean ± SEM) (%)	Blastocyst, n (mean ± SEM) (%)	Expanded blastocyst, n (mean ± SEM) (%)	Hatching blastocyst, n (mean ± SEM) (%)	Hatched blastocyst, n (mean ± SEM) (%)
37.0 ºC	0	16 (22.0 ± 4.9)	57 (78.0 ± 4.9)	0	0
38.5 ºC	1 (2.0 ± 1.8)	12 (21.0 ± 5.5)	43 (77.0 ± 5.7)	0	0
39.5 ºC	0	11 (18.0 ± 5.0)	49 (80.0 ± 5.1)	1 (2.0 ± 1.6)	0

Group	Quality A (mean ± SEM) (%)	Quality B (mean ± SEM) (%)	Quality C (mean ± SEM) (%)
37.0 ºC	58 (79.0 ± 4.8)	12 (16.0 ± 4.4)	3 (4.0 ± 2.3)
38.5 ºC	37 (66.0 ± 6.4)	16 (29.0 ± 6.1)	3 (5.0 ± 3.0)
39.5 ºC	48 (79.0 ± 5.3)	8 (13.0 ± 4.4)	5 (8.0 ± 3.5)

N: No. of putative zygotes. a, b: different letters in the same column indicate values statistically different ($P<0.05$).

5. DISCUSSION

Several studies point out toward the importance of mimicking *in vitro* the dynamic microenvironment provided by the oviduct and uterus during the encounter between oocytes and sperm and during the first steps taken by the zygotes towards the blastocyst stage. This microenvironment embraces important physic-chemical parameters such as pH, temperature, gas composition and oviductal and uterine secretions. All of them are capable of modulating gamete functions and their interactions as well as to control the embryonic development (Ghersevich et al., 2015). Recently, thanks to the advances in the reproductive and epigenetics fields, it is possible to study the impact of these factors during the demethylation-remethylation process performed at early embryo development. In addition, important changes have been identified in the porcine embryo gene expression during the *in vitro* culture, as a result of a deficient *in vitro* environment that could lead to severe consequence in embryos themselves and, therefore, in the new-borns that come from IVEP (Canovas et al., 2017a).

The epigenetic cost that undergo the *in vitro* embryos have become one of the main concerns for reproductive researchers due to the strong involvement of ART as treatments for infertile couples. For this reason, recently, many authors have performed several attempts to approximate the *in vitro* environment during ART to the physiological one with the purpose of reducing that epigenetic cost. This requires a detailed knowledge of the physiological environment where fertilization and embryo development take place in the female reproductive tract. For this reason, developing precise *in vivo* procedures to measure physiological parameters and transferring them to the *in vitro* conditions is a justified need.

For the validation of physiological parameters during ART, pig is an ideal research model. First, the pig has a high homology to humans, which has been powerfully demonstrated in genomic studies (Schachtschneider et al., 2015), being one of the most reliable animal models as information source. Second, its body size facilitates surgical procedures, and because pig is a multiparous animal, there can be obtained large numbers

of oocytes or embryos for research purposes. Finally, as mentioned, *in vitro* systems for embryo production in this species are severely deficient and therefore, an improvement of ART using physiological parameters could provide a new tool to increase the efficiency of these systems in this species. Ultimately, new information about a more physiological and bioinspired environment during ART would have important implications for agriculture and biomedical fields (Whyte & Prather, 2011).

The findings provided by the current study point out the need to approximate the pig IVEP conditions to those given *in vivo*. The studied approaches included in this work have tried to characterize physic-chemical parameters found within the oviductal and uterine environment of the female pig with the purpose of transferring them to the porcine IVEP system. These approaches, on the one hand, might be, in the case of pig, a starting point to bring closer the IVEP results of this species, in terms of efficiency, to those obtained in other livestock species, while in other species could be a further improvement of the current approaches. On the other hand, this approximation might be essential for the suitable molecular deployment during the conformation of a healthy embryo as epigenetic studies suggest.

Here, the results obtained with the approaches performed in this study, using the pig model, will be discussed, and compared with other approaches carried out by different researchers in porcine or other species.

The initial phase of the project was to evaluate the effect of adding oviductal and uterine proteins to the culture media during porcine IVEP, either through commercial proteins contained, in large amount, within the reproductive fluid (**experiment 1**) or through the addition of the own natural biofluids collected from oviducts and uteri (**experiment 2**).

The last phase of the project was to decipher the effect of physiological physic-chemical parameters such as oxygen (**experiment 3**) and temperature (**experiment 4**) on the outcome of the *in vitro* production of pig embryos. The novelty associated with these two experiments was the serial measurement of these parameters within the reproductive tract of female pigs with the aim of translating those results to pig ART. So far, it is the

first time that laparoendoscopic single site (LESS) surgery approach has been used in the pig for this purpose, with the big advantage of reducing intra-operative organ manipulation, post-operative pain and total recovery. In addition, the miniaturized luminescent probes used in this study allowed accurate and immediate determination of reliable oxygen and temperature levels while minimizing the insult to oviductal or uterine mucosa compared with previously performed procedures where laparotomy was used. Considering the multiple sexual conditions of the pig females currently used in farms and the variety in the source of oocytes used for ART and animal model studies, this work was carried out in two groups of pigs, one of pre-pubertal gilts treated for estrous cycle induction, and another one of sexually mature multiparous sows with no hormonal treatment. The first group resembles the oocytes used for IVM in the IVF laboratories, which stimulated to resume meiosis, whereas the second one could be considered as physiological.

5.1. EXPERIMENT 1. GAMETE CO-INCUBATION WITH EXOGENOUS OVIDUCTAL PROTEINS HARDENS THE ZP OF PORCINE OOCYTES REDUCING POLYSPERMY RATES DURING IVF

In this experiment, the effect of certain proteins contained in the porcine oviductal fluid from the periovulatory phase of the estrous cycle on the porcine ZP resistance to proteolysis it was evaluated. Ezrin, HSP-90α and HSP-70-1A were chosen and studied for their high abundance in the fluid responsible of a hardening effect. The data showed that oocytes incubated with each of these proteins had a more resistant ZP to pronase digestion compared to the control group (without any protein). Assuming these proteins are able to attach the ZP somehow, the simplest hypothesis about the mechanism by which these proteins would perform this hardening effect could involve the considerable molecular size of them, 70 kDa HSP-70-1A, 90 kDa HSP-90α (Jee, 2016) and 81 kDa ezrin (Gould, Cooper, Bretscher, & Hunter, 1986). Thus, these proteins could be creating a steric encumbrance by masking the binding sites for the enzyme on the ZP of the oocytes

because of its location and size. However, this hypothesis would not fully explain the consistent differences found between proteins. Therefore, it seems that these proteins could be more actively engaged in the process. For example, this hardening might be associated to the interaction of these proteins with the complex and three-dimensional ZP structure proposed by Jiménez-Movilla et al. (Jiménez-Movilla et al., 2009). In this way, the binding of the proteins to the ZP could induce changes in the tertiary structure of the glycoproteins that constitute the ZP itself, thereby affecting their three-dimensional position, and resulting in a higher packing, which would prevent temporarily the entrance of the enzyme to the site of proteolytic ruptures (Green, 1997). This assumption would be favoured by the idea of a specific protein-protein interaction previously described for the ZP hardening in the bovine species, involving the formation of disulphide bonds between the cysteine –SH groups of glycoproteins present in the oocyte's ZP (Iwamoto et al., 1999).

On the other hand, the ZP digestion time (ZPdt) in the oocytes incubated either with or without the different proteins was increased when heparin was added to the medium during the experiments. The stabilizing effect of heparin has been already shown when porcine oocytes were incubated with OVGP1 (Coy et al., 2008a). The presence of the common heparin binding motifs (LIGRK or LIGRR) (Hansen, O'Leary, Skubitz, Furcht, & McCarthy, 1995) within the protein's sequence would allow the binding of heparin to protein triggering a stronger hardening effect that would explain the observed results. As for heat-shock proteins, both references used in this study were full length human recombinant proteins produced in *E. coli* and HSP-70-1A contains the heparin-binding site LIGRK (73-77 aminoacid position) whereas HSP-90α does not. This could explain the hardening increase when HSP-70-1A was combined with heparin but the lack of this effect for HSP-90α. As for ezrin, although the molecule does not contain the classical heparin-binding sites, it has been shown that proteins from ezrin-radixin-moesin family can bind to heparin (Lankes, Griesmacher, Grünwald, Schwartz-Albiez, & Keller, 1988) and, specifically for ezrin, its N-terminal domain can bind to different glycosaminoglycans (Granés et al., 2003). Altogether, this would explain why HSP-70-

1A and ezrin induced a higher hardening when heparin was present and why this was not observed for HSP-90α.

It has been demonstrated that the capacity of oviductal fluid to induce pre-fertilization hardening in the ZP of oocytes from many livestock species is proportional to their ability to induce monospermy during IVF (Mondéjar et al., 2013). It also have been demonstrated that the hardening capacity of the oviductal fluid and hence, monospermy competence, relies upon its protein composition (Coy et al., 2008a). Therefore, in this experiment it was intended to verify whether the studied proteins with different hardening capacity, previously determined, are the molecules directly responsible for this monospermy proportions. The obtained data showed that proteins with higher hardening capacity had a higher monospermy rate. Although in this study, the hardening induced by these proteins was discrete, the changes occurred in the ZP seemed to be sufficient to be translated into a marked reduction of polyspermy rates. From these results, it could be concluded that the studied proteins have a major influence than the porcine oviductal pre-ovulatory fluid, since a discrete hardening effect has a large decrease on the sperm penetration. Nonetheless, when the hardening data obtained in the present work using the studied proteins, from one to two minutes, are compared with those obtained by Coy et al. (Coy et al., 2008b) using the oviductal pre-ovulatory fluid, around 4 hours, it can be concluded that other proteins present in this fluid exert a greater effect within the ZP hardening. In line with this last idea, here, it is proposed that changes in the ZP structure are not the unique responsible for the results on penetration and monospermy rates and it is possible that other changes at sperm membrane level or at the oocytes or even during their interactions, are important factors involved. Although the molecular mechanism by which these proteins reduce the number of sperm penetrated by oocyte has not been elucidated in this work, possible modes of action of these proteins, according to their main role within cells, can be hypothesized.

The reduction of the entrance of sperm in oocytes incubated with HSP proteins seems to be supported by changes in the ZP structure. This can be easily explained by the main role of HSP proteins in protein–protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein

aggregation (Borges, Fischer, Craievich, & Ramos, 2005). Therefore, this interaction could be occurring within the ZP structure during fertilization. Moreover, according to the results, it can be discarded that HSPs affect the recognition of the gametes since the number of sperm bound to the ZP was similar to the control group. Nevertheless, it cannot be discarded an effect of HSPs on sperm function. Lloyd et al. (2009) proposed the participation of the HSP-70-1A isoform in the maintenance of sperm viability in the oviduct. Moreover, the sperm motility reduction observed in the presence of a specific inhibitor of HSP-90 as well as after cryopreservation processes, in which HSP-90 levels were reduced, may indicate a relevant role of this last protein on sperm motility (Cao, Wang, Xiang, & Li, 2003; Huang et al., 2000).

The ZP is composed by a fibrous framework quite porous and permeable to relatively large macromolecules. Therefore, in the case of ezrin, which is normally a cytoplasmic protein, once added to the culture medium it could be able to penetrate inside the oocyte, through ZP porous, being activated and regulating the entrance of spermatozoa. Ezrin main function is to be a cross-linker between plasma membrane proteins and the actin cytoskeleton that involves the functional expression of membrane proteins at the cell surface. In this way, ezrin could enter to the cytoplasm of either sperm or oocyte and modify the plasma membrane, being able to regulate the massive entrance of spermatozoa to the oocyte. The reduction in the number of sperm bound to the ZP in presence of ezrin reinforces this belief but would not explain the significant hardening of the ZP when the oocytes were incubated with ezrin, compared to the other proteins. Therefore, it should not be discarded the idea of ezrin interacting with the ZP structure as well as ezrin affecting the sperm functionality. Piehl et al. (2013) described the presence of ezrin in boar exosomes present in seminal plasma, attributing it a stabilizing function to the cell membrane.

In order to determine whether sperm function was affected by the addition of different proteins during IVF, preliminary tests were performed in our laboratory, which are not detailed in this work. They included the incubation of sperm using different concentrations of the studied proteins at various times. The instrumentation used for analysing these tests varied, from microscopic examinations to the use of computer-

assisted sperm motility analysis or flow cytometry in conjunction with specific fluorescent probes for membrane and acrosome integrity. With these tests, on the one hand, sperm viability was observed and toxicity of the concentration of protein used as a cause for the decrease in penetration rates was ruled out; and, on the other hand, it was intended to know if they affected some parameters of sperm function as it was suggested by the above-mentioned authors. Preliminary tests showed that proteins did not affect sperm motility neither membrane or acrosome integrity, however, the use of different conditions and parameters would be needed to confirm these results. In addition, to contrast some of the hypotheses proposed above, an immunohistochemical study would be necessary, which would allow us to know the final location of the protein and to show where it binds in the oocyte. For instance, if the protein binds to the ZP, it would be participating in a mechanism of pre-fertilization hardening, if it enters to the ooplasm it could be participating in the membrane block of polyspermy, etc. In any case, knowing exactly the function of a protein is an arduous task due to the existence of a large number of complex molecular interactions that may interfere in its study.

From experiment 1 it is concluded then, that different proteins present in the oviductal pre-ovulatory fluid have different roles during fertilization. The addition of proteins contained within this fluid, separately, to the IVF culture medium have an impact on penetration and polyspermy rates but does not increase the final efficiency of the system. Therefore, the next step would be to ensure that all these proteins can operate effectively together as they do naturally and this is the main reason for what experiment 2 was developed.

5.2. EXPERIMENT 2. THE ADDITION OF REPRODUCTIVE FLUIDS TO THE SPERM SELECTION, IVF AND EC MEDIA IMPROVES IVEP OUTCOMES

The sperm quality and the prediction of fertility have always been a major concern for the clinical and agricultural andrologists. This is due to that, in contrast to the oocytes, which are more difficult to obtain, a seminal dose contains millions of spermatozoa being then an advantage the option of choosing, between them, the best ones to penetrate the

oocytes during IVF or intracytoplasmic sperm injection. Within the female reproductive tract, potentially fertile spermatozoa are selected. That sperm selection besides preventing polyspermy, also prepares and enables sperm cells for the fertilization process by means of fundamental physiological changes. Therefore, the importance of applying appropriate sperm selection procedures *in vitro* could also have a direct impact on the development of the future produced embryos.

In this experiment, the imitation of the natural environment during sperm selection was approached by using a migration procedure that avoids centrifugation, named swim-up, with the intention to overcome polyspermy during porcine IVEP. In addition, a further step was performed adding oviductal fluid from the pre-ovulatory phase of the estrous cycle to the sperm selection medium, trying to imitate the physiological changes incurred during sperm capacitation. These changes would be essential to enable sperm with the necessary competence to fertilize the oocytes and to develop good quality embryos successfully. The IVF efficiency of this new system was compared with the most known and extensively procedure used in pig, density gradient centrifugation. Our results show that the use of this new system improved the outcome of IVF. Besides, the addition of oviductal fluid to the selection medium was beneficial. Even though density gradient centrifugation using Percoll® is one of the most successful boar sperm selection methods since it achieves a considerable reduction of the alterations in morphology, motility and DNA impairment within sperm cells (Matás et al., 2011), the high rates of sperm penetrated per oocyte makes it a unpractical approach for the pig IVEP. Furthermore, centrifugation could lead to lethal damage in sperm (Alvarez et al., 1993) and it may result in an increment of the production of reactive oxygen species and sperm DNA hurt (Aitken & Clarkson, 1988) affecting then, the further development and production of healthy blastocysts. For this reason, it would be advisable to start searching for new alternatives. Swim-up seems to be a more natural procedure to be chosen for that purpose since it is the own spermatozoon who has to swim within a medium as it occurs in the female reproductive tract. Moreover, attending to a recent article where density gradient centrifugation and swim-up methods were compared by using human spermatozoa, density gradient centrifugation resulted in higher sperm concentration, whereas swim-up

resulted in spermatozoa with better quality in terms of morphology (Fácio, Previato, Machado-Paula, Matheus, & Araújo, 2016). In addition, this study is supported by previous articles, which concluded that density gradient centrifugation provides a greater semen sample from a quantitative point of view and hence, allowing the use of low quality samples, while swim-up is preferred from a qualitative perspective (Boomsma, Heineman, Cohlen, & Farquhar, 2004; Ricci et al., 2009).

In this work, high polyspermy rates were obtained with density gradient centrifugation compared to swim-up, as expected. High polyspermy rates after density gradient centrifugation appear to occur due to the large number of spermatozoa capacitated simultaneously and present at the site of fertilization (Dale & DeFelice, 2011). Sperm advanced capacitated status could be explained by the action of centrifugation, which would remove certain protective proteins exposing the sperm membrane to the fertilization medium where capacitation-related molecules such as calcium and bicarbonate would also accelerate the process. Thus, capacitation would take place in a far more progressive manner than in swim-up method, where capacitation may develop gradually. Following this hypothesis, with the swim-up methodology, the sequential arrival of sperm to the oocyte would be associated with monospermic penetration, since it would allow the oocyte to display an efficient blocking system. This last hypothesis would be supported by the idea of the different arrival timings of motile sperm during monospermic penetration reported by other authors (Funahashi et al., 2000; Li et al., 2003)

Park el al. (2009) used previously a modified swim-up method in pigs, which showed a higher reduction of polyspermy rates than those obtained in this work. Despite both swim-up methods have improved considerable the efficiency of IVF in relation to their control groups, the large differences between both procedures encourage us toward a deeper analysis described below. Here, it is suggested that these differences could be due to the modifications applied by the authors, both in relation to the IVF technique and the swim-up procedure used. On the one hand, Park et al. inseminated the oocytes in micro droplets instead of 500 μl wells, which suggest a more stable environment in the micro droplet during IVF; besides, they used a different IVF medium (Tris Buffer

Medium, TBM), whose composition could influence the results. On the other hand, they used a cell filter, a shorter distance for swimming (1 cm) and a longer time of incubation (1 h) during sperm selection. The use of a filter could result in a specific sperm selection while, on the contrary, shorter distance and longer time of incubation indicate a less strict selection process compared to that one carried out in this work. Nonetheless, we suggest that one of the fundamental differences responsible for these varied results is that they worked with frozen semen, thus avoiding the oscillations presented by the different ejaculates. This fact allowed them to choose the best boar for the production of *in vitro* embryos. In our laboratory swim-up protocol was optimized to obtain the best results using fresh sperm, which was opted in order to approximate the production of *in vitro* porcine blastocysts to that occurring in nature. Moreover, the optimization of swim-up using fresh ejaculates did not imply centrifugation before developing the technique because of the high initial proportion of motile sperm (more than 70 %) within these samples compared to the frozen samples used by Park et al., (Park et al., 2009) where a short centrifugation was necessary before and after swim-up. Apart from that, in this work the addition of oviductal pre-ovulatory fluid to the sperm selection medium with the purpose of obtaining *in vitro* embryos is a new approach that has not been tested before. Currently, in our laboratory, swim-up is being also optimized using frozen-thawed sperm, since it seems recommendable using in each replicate the same male for obtaining more stable results such as those reported by Park et al., (Park et al., 2009).

The data obtained with swim-up method in this study along with those reported by Park et al. (Park et al., 2009) show that the use of swim-up during sperm selection, which is not such a widely used method in pig, is the most reliable procedure for reducing polyspermy and improving the IVF efficiency in swine. However, what happens when putative zygotes obtained by these procedures are cultured until blastocyst stage?

A previous study in the bovine species reported the benefits of using oviductal fluid during EC on the development and quality of bovine embryos produced *in vitro* (Lopera-Vasquez et al., 2015). This study showed how low bovine oviductal fluid concentrations produced high quality bovine blastocysts, as reflected by their total mean number of cells per blastocyst, cryotolerance survival and gene expression data, while

higher bovine oviductal fluid concentrations seemed to have a negative effect on those parameters. For this reason, a new experiment was designed where two experimental groups were distinguished depending on whether or not the environment in which the porcine gametes and embryos were cultured contained porcine reproductive fluids as additives at every step of the IVF and EC procedures. The results showed that the addition of oviductal and uterine fluids to the culture media (obtained sequentially at the corresponding phases of the estrous cycle) improved the outcome of IVEP and the quality of the pig blastocysts produced *in vitro*.

In response to the question at the end of the previous subheading, it is worth to mention that the progression of 40% of the cleaved embryos to the blastocyst stage *in vitro* obtained in this study is indicating an improvement over the best previous results ever reported in swine of 30 – 35 % (Park et al., 2009). Even more, it was possible to obtain a higher yield (45 %) when the biofluids were added to the culture media. This achievement allows obtaining similar or even higher yields in the pig than in the bovine species. While Park et al., (2009) used a modified swim-up procedure obtaining high yield during pig IVF, these authors did not achieve significant improvements in the developmental potential of the porcine embryos compared to their control group, remaining below the obtained within this work. However, just as in this work, the quality of the produced blastocysts was enhanced, achieving 85 % of blastocysts produced with more than 50 cells in both works.

Other remarkable findings in this work were that blastocysts coming from the fluids group registered a more advanced developmental stage, and that the mean number of cells per blastocyst was higher than the blastocysts obtained in the control group. In addition, that number was the same as that obtained from blastocysts collected *in vivo*. This last result shows that the use of reproductive fluids as additives, even at the low dose used in this study (1%) is beneficial for improving considerably the quality of pig embryos bringing them close to their *in vivo* counterparts.

Due to the several studies that have demonstrated the negative impact of the *in vitro* conditions on the developing embryos, authors such as Blockeel et al. (2009) have

proposed the use culture devices to fertilize human oocytes and develop the human embryos in the uterus of women. These authors created a silicone culture device placed inside the uterus as a feasible and safe method to bring closer the quality of in *vitro* grown blastocysts to those obtained in natural conditions. Although practical, this solution dismisses the oviduct as an environment where fertilization takes place. According to the results obtained in the present work, it would not be necessary to insert a foreign material into the woman's body to bring human blastocyst *in vitro* to the quality of their *in vivo* counterparts; instead, it would be enough with adding a small amount of reproductive fluids to every step of the culture. In this scenario, both oviductal and uterine environment would be taken into account. Although the possibility of transferring these methods to the human clinic might seem far, the fact that nowadays other natural fluids such as breast milk for baby feeding or blood serum for transfusions are collected and stored at biobanks, make it possible to predict the future availability of human reproductive fluids obtained from oocyte donors during interventions at human infertility clinics. In fact, the first samples of these fluids are already stored at Biobanc-Mur in Spain (National Register of 433 Biobanks Nº B.0000859). On the other hand, reproductive fluids from different livestock species such as pig and bovine are being collected at EmbryoCloud (Murcia, Spain) in order to perform different studies that substantiate the use of human and animal reproductive fluids in the future.

5.3. EXPERIMENT 3. CULTURE UNDER THE PHYSIOLOGICAL OXYGEN MEASURED WITHIN THE REPRODUCTIVE TRACT OF FEMALE PIGS IMPROVES THE IVEP

The records of oxygen levels within the female reproductive tract of pig females displayed two potential profiles, flat or waved, which were not associated to any group of age or state of the estrous cycle, but influenced by the measuring site. Thus, no flat profile was observed in the uterus at any case. This finding suggests that there may be a different threshold for oxygen variation between the oviduct and uterus, which could be more restricted in the oviduct. A similar waved pattern was observed by Nichol et al. (1997) for the oviduct pH, which varied depending on the oviduct region (ampulla *versus* ampullary-isthmic-junction) and the stage of the estrous cycle (pre-, peri- and post-

ovulatory). Although these authors discarded a higher contractibility of the organ as the cause of the highly waved pH pattern this reason could not be ruled out in our study since the uterus always showed the waved pattern. As a general point, it has to be mentioned that the uterus is comprised of a powerful smooth muscle layer, the myometrium. This layer is able to contract itself at the right time during labour and remain relaxed during the period of pregnancy, but spontaneous contractions can be given at any time.

The major finding of this experiment was the significant differences found in oxygen levels within the reproductive organs between gilts and sows, which could be explained simply because younger animals have a higher metabolic rate (Martinez et al., 1981) and therefore, seems reasonable to think that oxygen levels could be higher within their organs. Nevertheless, the differences may also be due to the hormone treatment used in gilts to induce the estrous cycle. Changes in the gene expression profile of peri-implantation endometrium in gonadotropin-stimulated cycles compared with natural cycles have been reported (Mirkin, Nikas, Hsiu, Díaz, & Oehninger, 2004). Some of the genes affected are related to lipid and glucose metabolism, and might be responsible for changes in the demands of oxygen, explaining the differences between hormonal treated animals (gilts) and non-treated animals (sows). Therefore, the effect of gonadotropins should be considered in the interpretation of our *in vitro* culture (IVC) results as well. The use of gonadotropins for IVM or superovulation is very common and still necessary in both animals and humans. For example, *in vitro,* COCs are cultured for maturation in dishes the approximate time necessary for the oocytes to reach the Metaphase II stage as occurs within the ovarian follicles *in vivo*. Hormones (FSH and LH analogues) are added to the maturation media in order to mimic the natural events that take place within the ovarian follicles. During superovulation, elevated hormone concentrations are injected to the female for the production of multiple oocytes for their use in IVF.

Here, and according to our IVC results, we propose the use of physiological oxygen levels measured within the reproductive tract of sows (animals with non-hormonal treatment) throughout the IVF and EC period especially when using oocytes from young animals. Although a better embryonic development *in vitro* has been observed in oocytes collected from ovaries of adult females, compared with those of

prepuberal females (Marchal et al., 2001), prepuberal females are often used as oocyte source since the entrance of young animal groups into the slaughterhouses is higher (the average age for consumption is 6-8 months, depending on the countries). In addition, the use of prepuberal ovaries offers the added advantage of obtaining a larger number of oocytes for research purposes. However, this practise might lead precisely to the opposite effect of what is intend. Morphological criteria employed for standardization usually include the number of cumulus cell layers surrounding the oocytes and the uniformity observed in the cytoplasm´s aspect (Somfai et al., 2004), being them subjective criteria and insufficient, limited by the point of view of working personnel. Consequently, solutions to this regard should be provided as well. For this reason, the use of oxygen levels measured within the adult female (without hormonal treatment), it might be necessary to decrease the scale of a potential effect on the blastocyst yield caused by the use of gonadotropins either to induce ovulation for the *in vivo* collection of oocytes or during IVM of oocytes, especially when they are coming from pre-pubertal animals. Additional studies using low oxygen concentration during human IVF would help to clarify this hypothesis.

In this study, the establishment of pig oxygen values at different sites in the same organ was performed for the first time. In the case of the oviduct, the isthmus plays an important role as reservoir for sperm (Suarez, Revah, Lo, & Kölle, 1998) while the ampulla is the place where fertilization occurs (Kölle et al., 2009). For this reason, oxygen data recorded in those sites have a special relevance. However, no differences in oxygen tension were found between both sites, neither between reproductive states, but remarkably, a tendency of higher oxygen concentration when pigs are approaching the pre-ovulatory phase was observed. The oxygen peak observed in this study matches with the volume increment of oviductal fluid during the pre-ovulatory phase caused by the increase of steroid hormones, mainly estrogen (Hunter et al., 1983). In addition, it corresponds to the highest expression levels of the vascular endothelial growth factor receptor-two (VEGF-R2 or KDR) observed in the oviduct mucosa of the pig (López Albors et al., 2017). Binding between VEGF-R2 and its activating factor VEGF-A is assumed to enhance the effect of VEGF-A on the vascular permeability of the oviductal

epithelium. Furthermore, enzymes involved in steroid biosynthesis require oxygen molecules (Payne & Hales, 2004). Hence, an interaction between steroid hormones, the factors regulating vascular oviduct permeability, and oxygen is evoked from our results.

Nowadays, while the use of low oxygen tension (5 %) during embryo culture take part as a standardized protocol performed by all human fertility clinics during *in vitro* embryo culture, in traditional IVF, pig embryos are still exposed to a gas phase of atmospheric (20%) oxygen. In addition, oxygen concentration has not been as well established in animal reproduction centres, where low oxygen environment (5 %) is more prevalent only in ruminants during ART.

Preliminary *in vitro* studies in animals have reported the positive effect of using 5 % oxygen on IVEP, as those reported in the bovine species by Lim et al. (Lim, Mei, Chen, Godke, & Hansel, 1999), where an artificial and defined culture media (modified Bovine Embryo Culture Medium mBECM) was used. This work also proved that the combination of low oxygen with the addition of a biological protein source such as Fetal Bovine Serum (FBS) to the culture media provides a more suitable atmosphere. In this study, we used oviductal and uterine fluids to provide a physiological environment. In the case of pig, Berthelot and Terqui (Berthelot & Terqui, 1996) and Im et al. (Im et al., 2004) have concluded that the best oxygen tension to improve the EC conditions is 5 %. In the first study, 2, 5, 10 and 21 % oxygen were compared, but the *in vivo* value (7%) recorded in our study was not used in those studies. In the second study, 5 and 21 % oxygen concentrations were compared by using porcine embryos generated by nuclear transfer. However, the most important difference of our study relative to these preliminary studies is that a physiological oxygen tension was recorded for first time in pigs, which have not been used before, and was adopted during both IVF and EC periods. Although 5 % oxygen is closer to the physiological values than 20 %, human embryos for instance, are exposed only to a 1.5 % oxygen atmosphere in the uterus (Ottosen et al., 2006) and oxygen tension in the fallopian tubes remains unknown due to the difficulty in obtaining these values. Therefore, it is unknown whether a value even lower, and more similar to the physiological one, could be more beneficial. Up to now, most published experiments including IVF and embryo culture in pigs are carried out under atmospheric oxygen

conditions (20 %). However, the only study in swine combining low oxygen levels (8-10%) during both IVF and EC has shown a beneficial developmental effect on porcine blastocysts (Karja et al., 2004). Despite these data, it was unknown until now the precise oxygen tension in the swine reproductive tract and whether the use of these values might improve the efficiency of porcine *in vitro* embryo production systems.

From the present data, a larger number of pig blastocysts and an increase in their mean number of cells were obtained when embryos were fertilized and cultured under the oxygen tension (7 %) found in the reproductive tract of sows compared to conventional oxygen tension (20 %). The results suggest that embryos fertilized and cultured under hypoxia conditions have a tendency to achieve a more advanced embryonic stage regarding to the time. This beneficial effect may be associated with a reduction of metabolic stress that leads to a decrease of ROS, DNA fragmentation and finally, apoptosis (Agarwal, Saleh, & Bedaiwy, 2003; Kitagawa et al., 2004), although these hypotheses should be further tested and remain speculative at the moment.

5.4. EXPERIMENT 4. CULTURE UNDER THE PHYSIOLOGICAL TEMPERATURE GRADIENT FOUND WITHIN THE REPRODUCTIVE TRACT OF FEMALE PIGS IMPROVES THE BLASTOCYSTS YIELD IN VITRO

Temperature is a key parameter in physiological, behavioural and ecological research in mammals. However, its measurement within internal organs becomes a challenge since temperature variations can be easily given in response to external environmental parameters. Hence, it is of vital importance the use of minimal invasive procedures, as those used in this work, to measure the temperature within the reproductive tract of females. Even so, the values registered as low as 35.8 ºC and as high as 41.3 ºC in a few animals of the present study may be due to the anaesthesia or manipulation of the organs and therefore, and they might not have a biological meaning.

The major finding of this experiment was the significant differences found in temperature between the oviduct and the uterus in sows. While Hunter and Nilchol (Hunter & Nichol, 1986) found a temperature gradient of 1.5 ºC between the ampulla and

isthmus, this was not observed in the present study. Our study supports the recent data from Hino and Yanagimachi in mice (Hino & Yanagimachi, 2019) claiming that peristaltic movement within the oviduct and the continue ad-ovarian transport of oviductal fluid make a temperature gradient within the oviduct unlikely existent. In contrast, we found a gradient of 1.5 ºC between the oviduct and uterus for first time. This gradient involved a reduction in the temperature within the oviduct, remarking a possible function of low temperature (37.0 ºC) during pig fertilization. To highlight the importance of this finding, this gradient was transferred to *in vitro* conditions and was studied.

On the other hand, although no significant, it was found a trend towards higher temperature (1.0 ºC) at the peri-ovulatory (PO) and luteal (LU) stages in sows. This could be attributed to the increment of progesterone concentrations in blood, which has a role in the warming of body temperature (Barron & Fehring, 2005) and hence, it might lead to the temperature increment of the reproductive organs. In addition, prior research suggests that reproductive hormones participate in the thermoregulation of different tissues (Nakayama, Suzuki, & Ishizuka, 1975; Silva & Boulant, 1986; Stachenfeld, Silva, & Keefe, 2000) and therefore, steroid hormone administration in gilts may be the cause for not observing temperature variations within the reproductive organs of gilts.

According to fluid-mosaic membrane model proposed by Singer and Nicholson in 1972 (Singer & Nicolson, 1972), sperm membrane is organized as the rest of the cell membranes in an asymmetric bilayer of phospholipids with the hydrophobic chains arranged internally and the hydrophilic heads externally, and between them, cholesterol and different proteins are found. The degree of fluidity of the membrane depends on the degree of the acyl chain length saturation, the amount of cholesterol or the temperature. The lipids within the bilayers undergo radical changes in physical state at a characteristic phase transition temperature (Mantsch & McElhaney, 1991). Below that transition temperature, fluid lipids freeze, and above that temperature, lipids become more fluid. According to our results, the range of temperature transition phase for the boar sperm membrane (35.5 - 40.9 ºC) matches with the different temperature variations found within the reproductive tract of the female pig. Most significant of all, however, is that the peak temperature considered the temperature transition phase for capacitated boar sperm

membrane (37.1 ºC) coincides with the temperature found within the oviduct of the female pig indicating that this temperature is relevant during the fertilization process.

More than 20 years after the fluid-mosaic membrane model, it was published the lipid raft model, which suggested that specific areas of the cell membrane with specific lipid and protein composition (microdomains or lipid rafts) move over a fluid bilayer; and that the interactions between microdomains are responsible of signalling transduction in cells (Simons & Ikonen, 1997). The overcome of fertilization depends partially on the reorganization of microdomains in the sperm head surface during capacitation, which are involved in important biological activities such as sperm zona pellucida binding, acrosome reaction and sperm-oocyte recognition (Boerke, Tsai, Garcia-Gil, Brewis, & Gadella, 2008). During sperm capacitation, membranes undergo a deep rearrangement that affect the composition and the biophysical properties of the cell membrane and these microdomains: membrane fluidity increases, lipids displace from one leaf to the other one, even within the same leaf; and the activity of several enzymes is modulated. This work focuses mainly on the effect of temperature on membrane fluidity in sperm and its impact on fertilization. The strong correlation found between the calculated coefficient diffusion (CDC) of the lipophilic fluorescent dye and temperature ($r = - 0.96$) indicates that temperature induces membrane fluidity changes significantly at the sperm surface and hence, it can be assumed that temperature participates in the reorganization of these microdomains. Surprisingly, this correlation is negative, which means that the higher the temperature, the lower the CDC. While it is true that according to the fluid-mosaic membrane model the exposition of sperm membrane to temperature higher than temperature transition should increase lipid fluidity, the results indicate that sperm membranes are able to adapt to the environment, possibly changing the expression of intracellular pathways, adapting membranes to preserve homeostasis. One of the reasons of this fact may be to protect sperm against possible stressors such as extreme temperatures.

The results from FRAP analysis shows how the CDC of the lipophilic fluorescent dye increases from the control group (sperm at room temperature) to the sperm incubated at 37.0 ºC in agreement with the mosaic fluid model. However, when sperm are incubated

at higher temperatures, especially at 39.5 ºC, the CDC of the lipophilic fluorescent dye begins to decrease, that might have a negative impact on fertilization. In addition, different incubation temperatures during sperm capacitation involve the emergence of specific sperm subpopulations with a different functional status. While two populations where found in sperm incubated at 37.0 ºC (transition temperature), which may have a relevant role during fertilization, an intermediate subpopulation was observed when sperm were incubated at higher temperatures (38.5 and 39.5 ºC). This last could be in consonance with homeostasis mechanisms.

Nowadays, only one temperature (body temperature) is used during the different steps of IVEP as a standardized protocol performed by all human fertility clinics and animal reproduction centres. However, a few authors that have measured *in vivo* the temperature within the reproductive organs in different species have found minimal temperature variations (1 – 2 ºC) between these organs, even inside the same organ, suggesting a relevant role of these variations during the reproductive process (Hunter, 2012b)

From the results obtained in this experiment, a large number of blastocysts was obtained when the temperature gradient found *in vivo* was imitated in *in vitro* conditions, in other words, when oocytes were inseminated at the temperature measured within the oviduct (37.0 ºC) and putative zygotes cultured at the temperature recorded within the uterus (38.5 ºC), compared to the control group widely used in reproduction centres (temperature always set at 38.5 ºC). In addition, according to the IVF results, putative zygotes cultured at 37.0 ºC showed the lowest mean number of spermatozoa penetrated per oocyte, suggesting a higher incidence of euploidy of the embryos produced at this temperature.

Nevertheless, an additional group was studied in this work, which can be considered the opposite to the group at 37.0 ºC mentioned above, since a higher temperature (39.5 ºC) was used. When IVF outcomes were analysed in deep for all the groups, the higher penetration and polyspermy rates showed for the group at 39.5 ºC and the higher monospermy observed for the group at 37.0 ºC could be explained by the effect

of temperature on the differentiation of sperm subpopulations found in this experiment. At the time of fertilization, the way in which the different sperm subpopulations interact with the oocytes for each group could determine the outcome of fertilization. Therefore, the sperm membrane fluidity, which is affected by temperature, may be involved in the acquisition of fertilizing ability. Due to the fact that 39.5 ºC group had the highest mean number of spermatozoa penetrated per oocyte, the higher cleavage rate observed in this group later during EC seems to indicate a higher blastomere fragmentation. This fragmentation is generally considered as indicative of poor embryo quality in mammals (Puissant, Van Rysselberge, Barlow, Deweze, & Leroy, 1987). Therefore, the use of a temperature of 39.5 ºC during ART in pig appears to be a stressful factor for sperm. Then, the outcome of the results obtained in this work highlights the importance of using suitable temperature variations for mitigating the detrimental effect produced by the *in vitro* culture.

Once a detailed information of *in vivo* measures in the different stages of estrus cycle and organs have been provided by this PhD thesis, further studies with a wider group of experiments using together the physiological oxygen and temperature levels registered as well as the use of reproductive fluids at each step of the IVEP would be necessary. All the parameters found in reproductive organs and studied in this Thesis are important modulators of the microenvironment in which gametes and embryos develop and thus, there should be transferred to ART since the damage that non-natural conditions might cause to the embryo remains still unknown. With the results of this work, it has been demonstrated in terms of embryo development and quality, that the use of female reproductive fluids and the same oxygen and temperature conditions as those found in the reproductive organs during each step of the process (IVF and EC) is more beneficial for the production of embryos than the protocols used up to now.

6. CONCLUSIONS

1. The addition of exogenous proteins, detected within the porcine oviductal fluid, to the fertilization media reduces the penetration and polyspermy rates on pig *in vitro* fertilization through the hardening of the oocytes' zona pellucida but it does not improve the global *in vitro* fertilization outcomes.

2. The addition of porcine reproductive fluids obtained sequentially at the corresponding phases of the estrous cycle, to the sperm selection, fertilization and embryo culture media improves the *in vitro* embryo production outcomes and brings closer the quality of the blastocysts produced, in term of cell number, to their *in vivo* counterparts.

3. Culture under the physiological oxygen tension measured within the reproductive tract of adult female pigs improves the efficiency of the porcine *in vitro* embryo production system and enhances the quality of the blastocysts produced.

4. Culture under the physiological temperature gradient between the oviduct and uterus, measured within the reproductive tract of female pigs, reduces polyspermy during *in vitro* fertilization through the sperm membrane reorganization thus, improving the *in vitro* embryo production outcomes.

ABBREVIATIONS

AI: artificial insemination

ART: assisted reproductive technologies

BSA: bovine serum albumin

BTS: Beltsville Thawing Solution

CAI: Intracervical insemination

CDC: calculated diffusion coefficient

COCs: cumulus-oocyte complexes

CTAB: cetyltrimethylammonium bromide

dbcAMP: dibutyryl cyclic adenosine monophosphate

DPBS: Dulbecco's phosphate-buffered saline

DSC: differential scanning calorimetry

EC: embryo culture

eCG: equine chorionic gonadotropin

EDTA: Ethylenediamine tetra-acetic acid

EF: early follicular

ESHRE: European Society of Human Reproduction and Embryology

ET: embryo transfer

FAF: fatty acids-free

HSPs: heat shock proteins

IVC: *in vitro* culture

IVEP: *in vitro* embryo production

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

LESS: laparo-endoscopic single-site surgery

LU: luteal

MPN: male pronucleous formation

NA: not available

NCSU: North Carolina State University

OF: oviductal fluid

OVGP1: oviductin

PO: peri-ovulatory

POF: porcine oviductal fluid

PUF: porcine uterine fluid

PVA: polyvinyl alcohol

ROS: Reactive Oxygen Species

RT: room temperature

FAO: Food and Agriculture Organization

FBS: foetal bovine serum

FF: follicular fluid

FRAP: fluorescence recovery after photobleaching

hCG: human chorionic gonadotropin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIFs: hypoxia-inducible factors

hpi: hours post-insemination

SEM: standard error of the mean

S/O: spermatozoa per oocyte

SS: saline solution

TALP: tyrodes's albumin lactate pyruvate medium

TCM: Tissue Culture Medium

ZP: zona pellucida

ZPb: spermatozoa bound to the zona pellucida

ZPdt: zona pellucida digestion time

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