Universitat de Girona

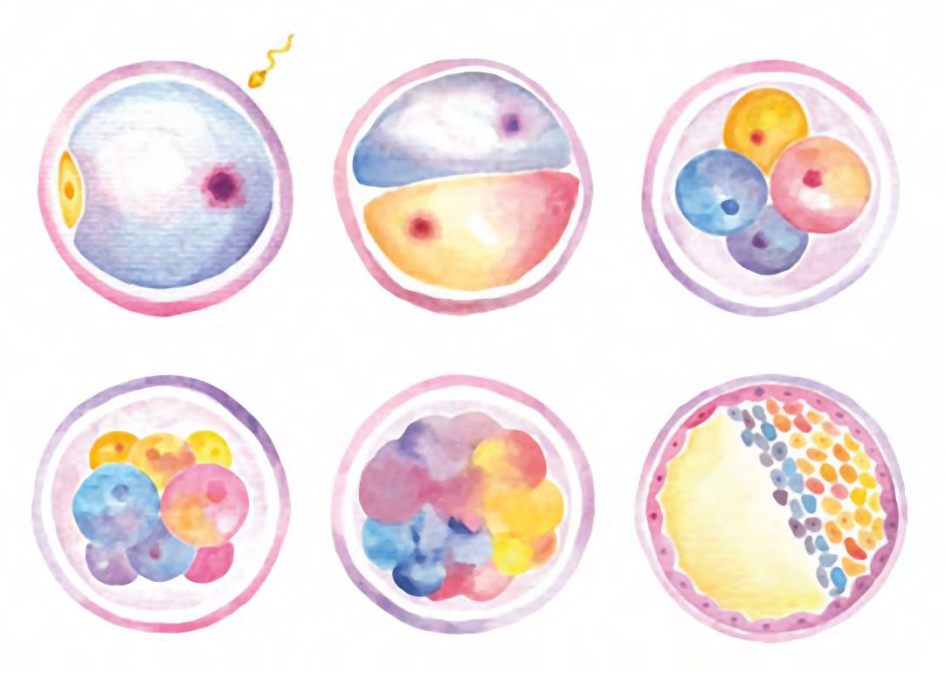
NOVEL INSIGHTS INTO PATERNAL FACTORS INFLUENCING THE MATERNAL ENVIRONMENT AND EMBRYO DEVELOPMENT

Yentel Mateo Otero

Universitat de Girona

DOCTORAL THESIS

Novel insights into paternal factors influencing the maternal environment and embryo development



Yentel Mateo Otero

2023

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Novel insights into paternal factors influencing the maternal environment and embryo development

Yentel Mateo Otero

2023

Doctoral programme in Technology

Supervised by:

Dr. Marc Yeste Oliveras

Dra. Isabel Barranco Cascales

Thesis Dissertation submitted to obtain the degree of PhD at the University of Girona

Universitat de Girona

Dr Marc Yeste Oliveras, Associate Professor of Cell Biology at the Department of Biology, University of Girona,

Dra Isabel Barranco Cascales, Ramón y Cajal Researcher at the Department of Medicine and Animal Surgery, University of Murcia,

DECLARE:

That the thesis entitled "*Novel insights into paternal factors influencing the maternal environment and embryo development*", submitted by Miss. Yentel Mateo Otero to obtain the doctoral degree, has been completed under our supervision and meets the requirements for the International Doctorate mention.

And for all intents and purposes, we hereby sign this document.

Signatures

Girona, 19th April 2023

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA
DEPARTMENT OF VETERINARY MEDICAL SCIENCES

Bologna, October the 4th 2021

To whom it may concern,

The present is to certify that Yentel Mateo Otero, from the University of Girona, Spain, has followed a 3-month period (July 1st to October 1st) of training and research in our laboratory at University of Bologna, Italy. She has successfully learned oocyte maturation, in vitro fertilisation, and embryo culture under my supervision. Mrs Mateo Otero has highly shown interest and dedication to her tasks, and she has demonstrated ability to participate in scientific discussions, methodological and/or of specific biological value. In addition, she has demonstrated the capacity to work in a team and proactiveness, a real asset when joining groups outside of the core graduate environment.
I express full satisfaction for the activities and capacities demonstrated during this stay.

Sincerely
Prof Diego Bucci
Veterinary physiology
University of Bologna

DEPARTMENT OF PHYSIOLOGY, ANATOMY AND GENETICS

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PERSONNEL/CONFIDENTIAL

30 May 2022

Yentel Mateo Otero
Department of Physiology, Anatomy and Genetics
C/o Sherrington Building

To whom it may concern,

RE: Yentel Mateo Otero

Please accept this letter as confirmation that Yentel has completed their visit at the Department of Physiology, Anatomy and Genetics at the University of Oxford in the Srinivas group. Their visit commenced on 01 February 2022 and ended on 30 June 2022.

Please do not hesitate to contact me if you should have further queries.

Yours sincerely,

Ellie Bonthorne
HR & Payroll Assistant

“Alice: Would you tell me, please, which way I ought to go from here?

The Cheshire Cat: That depends a good deal on where you want to get to.

Alice: I don't much care where.

The Cheshire Cat: Then it doesn't much matter which way you go.

Alice: ...So as long as I get somewhere.”

Lewis Carroll. Alice in Wonderland

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List of Publications

The present Dissertation is presented as a compendium of 6 publications:

Paper I

Mateo-Otero Y, Sánchez JM, Recuero S, Bagés-Arnal S, McDonald M, Kenny DA, Yeste M, Lonergan P, Fernandez-Fuertes B. **Effect of Exposure to Seminal Plasma Through Natural Mating in Cattle on Conceptus Length and Gene Expression.** *Front Cell Dev Biol* 2020;8:341 (doi: 10.3389/fcell.2020.00341).

- **Impact Factor:** 6.684
- **JCR Category**: Developmental biology / Cell Biology
- **JIF Quartile**: Q1

Paper II

Mateo-Otero Y, Viñolas-Vergés E, Llavanera M, Ribas-Maynou J, Roca J, Yeste M, Barranco I. **Aldose Reductase B1 in Pig Seminal Plasma: Identification, Localization in Reproductive Tissues, and Relationship With Quality and Sperm Preservation.** *Front Cell Dev Biol* 2021;9:683199 (doi: 10.3389/fcell.2021.683199).

- **Impact Factor**: 6.684
- **JCR Category**: Developmental biology / Cell Biology
- **JIF Quartile**: Q1

Paper III

Mateo-Otero Y, Fernández-López P, Delgado-Bermúdez A, Nolis P, Roca J, Miró J, Barranco I, Yeste M. **Metabolomic fingerprinting of pig seminal plasma identifies *in vivo* fertility biomarkers.** *J Anim Sci Biotechnol* 2021;12:113 (doi: 10.1186/s40104-021-00636-5).

- **Impact Factor**: 6.175
- **JCR Category**: Agriculture, Dairy & Animal Science
- **JIF Quartile**: Q1

Paper IV

Mateo-Otero Y, Ribas-Maynou J, Delgado-Bermúdez A, Llavanera M, Recuero S, Barranco I, Yeste M. **Aldose Reductase B1 in Pig Sperm Is Related to Their Function and Fertilizing Ability.** *Front Endocrinol* 2022;13:773249 (doi: 10.3389/fendo.2022.773249).

- **Impact factor (2021)**: 6.055
- **JCR Category**: Endocrinology & Metabolism
- **JIF Quartile**: Q1

Paper V

Mateo-Otero Y, Madrid-Gambin F, Llavanera M, Gomez-Gomez A, Haro N, Pozo OJ, Yeste M. **Sperm physiology and *in vitro* fertilising ability rely on basal metabolic activity: insights from the pig model**. *Commun Biol* 2023;6:344 (doi: 10.1038/s42003-023-04715-3).

- **Impact factor (2021)**: 6.548
- **JCR Category**: Biology
- **JIF Quartile**: Q1

Paper VI

Mateo-Otero Y, Llavanera M, Recuero S, Delgado-Bermúdez A, Barranco I, Ribas-Maynou J, Yeste M. **Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs.** *Biol Res* 2022;55:15 (doi: 10.1186/s40659-022-00386-2).

- **Impact factor (2021)**: 7.634
- **JCR Category**: Biology
- **JIF Quartile**: Q1 (D1)

Abbreviations

ADAM	A Disintegrin And Metalloprotease
AI	Artificial Insemination
AKR	Aldo-Keto Reductase
AKR1B1	Aldose Reductase B1
BSP	Bovine Seminal Plasma Proteins
β-NGF	β-Nerve Growth Factor
CALM1	Calmodulin 1
cAMP	Cyclic Adenosine Monophosphate
CG	Chorionic Gonadotropin
CITED1	Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxyterminal
CL	Corpus Luteum
COC	Cumulus-Oocyte Complex
CSF	Colony-Stimulating Factor
DAG	Diacylglycerol
DLD	Dihydrolipoamide Dehydrogenase
E2	Oestrogen
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Extracellular Vesicles
FSH	Follicle Stimulating Hormone

GnRH	Gonadotrophin Releasing Hormone
HNRNPDL	Heterogeneous Nuclear Ribonucleoprotein D Like
HPG	Hypothalamus-Pituitary-Gonad Axis
ICM	Inner Cell Mass
ICSI	Intra-Cytoplasmatic Sperm Injection
IFN-T	Interferon Tau
IL	Interleukin
IP_3	Inositol Triphosphate
IVC	*In vitro* Embryo Culture
IVF	*In vitro* Fertilisation
IZUMO1	Izumo Sperm-Oocyte Fusion 1
JUNO	Folate Receptor 4
LC-MS/MS	Liquid Chromatography Coupled With Tandem Mass Spectrometry
LDH	Lactate Dehydrogenase
LH	Luteinising Hormone
LIF	Leukemia Inhibitory Factor
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time of Flight
MAR	Matrix Attachment Region
MIF	Migration Inhibitory Factor
MPO	Myeloperoxidase

MS	Mass Spectrometry
NE	Neutrophil Elastase
NET	Neutrophil Extracellular Traps
NMR	Nuclear Magnetic Resonance
Oxphos	Oxidative Phosphorylation
P4	Progesterone
PGC	Primordial Germ Cells
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PIP_2	Phosphotidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCζ	1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Zeta-1
PSP	Porcine Seminal Plasma Proteins
PTGS2	Prostaglandin-Endoperoxide Synthase 2
ROS	Reactive Oxygen Species
ROC	Receiver Operating Characteristic
SACY	Soluble Adenylyl Cyclase
SCD	Sperm Chromatin Dispersion test
SCSA	Sperm Chromatin Structure Assay
SP	Seminal Plasma
SPACA6	Sperm Acrosome Membrane-Associated Protein 6

SRF	Sperm Rich Fraction
SRF-P1	First Portion (first 10 mL) of the SRF
SRF-P2	Second Portion (remaining volume) of the SRF
TE	Trophectoderm
TFGB3	Transforming Growth Factor Beta 3
TMEM95	Transmembrane Protein 95
TNF	Tumour Necrosis Factor
TRAIL	TFN-Related Apoptosis Inducing Ligand
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
VEGF	Vascular Endothelial Growth Factor
ZP	Zona Pellucida

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Abstract

For many years, reproductive outcomes were considered as being solely determined by the genome of oocytes and sperm. Yet, in the last decades, many other female and male factors have also been found to be relevant. Focusing on the latter, paternal factors have been proposed to be capable of modulating multiple features of the reproductive process, such as sperm physiology, the maternal environment and, even, the offspring health. Considering that the molecular mechanisms underlying this paternal regulation are mostly unknown, the objective of the present Dissertation was to increase the current knowledge on the role played by seminal plasma (SP) and sperm on the reproductive success, using bovine and porcine as animal models. For this purpose, Chapter 1 sought to investigate the involvement of SP in the modulation of *in vivo* fertility, and the potential pathways behind this regulation. In particular, the first work included in Chapter 1 explored whether uterine exposure to SP improved embryo survival and development in cattle. The results of this study demonstrated that while bovine SP did not elicit changes either in corpus luteum volume and weight or in embryo survival, embryos recovered from SP-primed uteri were longer than those of the control group and also differed in the expression of several embryo developmental biomarkers, including *CALM1*, *CITED1*, *DLD*, *HNRNPDL*, *PTGS2*, and *TGFB3*. These data suggested that SP was able to evoke changes in the female reproductive environment, with a positive effect on early embryo development. Thus, the following studies examined the putative molecular mechanisms contributing to this regulation, including the protein and metabolite composition of SP. Accordingly, the second work of Chapter 1 aimed to clarify whether and how the proteins present in the SP modulate *in vivo* fertility, using Aldose Reductase B1 (AKR1B1) as a candidate because of its previously reported positive association with increased farrowing rates in pigs. Data from this research showed that the first 10 mL of the sperm-rich fraction, which

contain sperm with the best reproductive traits, had the lowest concentration of AKR1B1 in SP (SP-AKR1B1). In addition, no relationship between SP-AKR1B1 levels and any of the sperm quality and functionality parameters evaluated upon arrival to the laboratory or after 72 h of liquid storage was observed. These findings allowed conjecturing that the positive influence of SP-AKR1B1 on *in vivo* fertility could be attributed to the modulation of embryo development and/or the female reproductive tract through the synthesis of certain prostaglandins, rather than to the effects of this protein on sperm physiology. Finally, the last work of Chapter 1 intended to evaluate the potential relationship between SP metabolite composition and *in vivo* fertility outcomes in pigs, in terms of farrowing rate, litter size, stillbirths per litter and pregnancy duration. From the 24 metabolites identified and quantified in all SP-samples, 12 of them were found to be related to specific *in vivo* fertility parameters. Specifically, this study showed that: i) lactate concentration in SP was associated to farrowing rate; ii) carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate and glucose concentrations in SP were linked to litter size; iii) citrate, creatine, malonate, phenylalanine and tyrosine concentrations in SP were related to the number of stillbirths per litter; and iv) malonate and fumarate concentration in SP were associated to gestation duration. Although the literature previously reported that most of these SP metabolites regulate sperm physiology, their effects on the female reproductive tract have not been investigated. Hence, further research should interrogate through which exact mechanisms these SP metabolites influence fertility. On the other hand, Chapter 2 sought to determine to which extent sperm components, including proteins, DNA and metabolites, can shape *in vitro* fertility outcomes, particularly oocyte fertilisation and pre-implantation embryo development. Particularly, the first work of Chapter 2 investigated whether sperm AKR1B1 was related to sperm physiology and *in vitro* fertilisation (IVF) outcomes in pigs. The results showed that the levels of the potentially inactive sperm AKR1B1 were

negatively associated to sperm motility and intracellular calcium levels, which are parameters linked to premature capacitation. In addition, higher levels of the potentially inactive AKR1B1 form were associated to lower IVF outcomes, both in terms of oocyte fertilisation and embryo development. Based on these data, one could suggest that the AKR1B1 present in sperm is involved in the regulation of sperm fertilising ability. Next, given the relationship between specific energy-related SP metabolites and *in vivo* fertility outcomes identified in the third work of Chapter 1, the second study included in Chapter 2 analysed the relationship between sperm metabolism and IVF outcomes in pigs. The results showed that high-quality sperm samples were associated to greater levels of glycolysis-derived metabolites, suggesting that pig sperm use glycolysis as the main catabolic pathway. Interestingly, sperm preferably utilising glycolysis were linked to a higher percentage of embryos at day 6. These data supported that sperm metabolism could influence IVF outcomes. The relevance of both sperm metabolism and AKR1B1 levels in sperm were hypothesised to be explained by reactive oxygen species, which are known to induce DNA damage. Hence, the last work of Chapter 2 investigated whether sperm DNA breaks could influence IVF outcomes in pigs. The results revealed that DNA breaks compromised embryo development, but not sperm fertilising ability. Indeed, global and double-strand DNA breaks were found to negatively affect the percentage of early blastocysts/blastocysts and the percentage of hatching/hatched blastocysts. In addition, the developmental potential of morulae at day 6 was also seen to be negatively influenced by the incidence of double-strand DNA breaks. All these results evidenced that sperm DNA damage may delay embryo development and even promote developmental arrest at early embryo stages. In conclusion, the findings of this Dissertation indicated that seminal factors can directly and indirectly modulate the maternal environment and influence fertilisation and embryo development. Further research should be focused on comprehensively determining the

male-female-embryo cross-talk to better understand which factors underlie the reproductive success in mammals.

Resum

Durant força temps, el paradigma dominant en l'àmbit de la Biologia de la Reproducció ha considerat que l'èxit reproductiu depèn únicament del genoma dels oòcits i els espermatozoides. Tanmateix, en les últimes dècades, s'ha descrit la rellevància d'altres factors, tant femenins com masculins. Concretament, s'ha observat que aquests darrers són capaços de modular diferents aspectes essencials del procés reproductiu, com ara la fisiologia espermàtica, l'ambient matern i, fins i tot, la salut de la descendència. Tenint en compte que els mecanismes moleculars subjacents a aquesta regulació no es coneixen amb profunditat, l'objectiu d'aquesta Tesi Doctoral va ser determinar el paper del plasma seminal (PS) i els espermatozoides en l'èxit reproductiu, emprant les especies bovina i porcina com a animals model. Amb aquesta finalitat, en el Capítol 1 es va investigar si el PS afectava d'alguna manera la fertilitat *in vivo*, així com les vies de senyalització que hi podrien estar involucrades. En particular, el primer treball inclòs en el Capítol 1 va indagar si la exposició uterina al PS millorava la supervivència i desenvolupament embrionari en boví. Els resultats d'aquest estudi van demostrar que, si bé el PS boví no va provocar canvis en el volum i pes del cos luti ni en la supervivència embrionària, els embrions recuperats dels úters exposats al PS eren més llargs que els del grup control i diferien en l'expressió de varis gens de desenvolupament embrionari, inclosos els següents: *CALM1*, *CITED1*, *DLD*, *HNRNPDL*, *PTGS2*, i *TGFB3*. Aquestes dades van suggerir que el PS podria provocar canvis en l'ambient matern, la qual cosa tindria un efecte positiu sobre el desenvolupament embrionari primerenc. En conseqüència, els següents estudis van examinar els mecanismes moleculars que podrien contribuir a aquesta regulació i que podrien involucrar les proteïnes i els metabòlits del PS. D'aquesta manera, el segon treball del Capítol 1 tenia com a objectiu esclarir si les proteïnes presents en el PS podrien regular la fertilitat *in vivo*, utilitzant la aldosa reductasa B1 (AKR1B1) com a proteïna candidata degut

a la seva associació positiva amb el rendiment reproductiu descrita prèviament a l'espècie porcina. Les dades d'aquest treball van mostrar que els primer 10 mL de la fracció rica en espermatozoides, fracció que conté els espermatozoides amb millors trets reproductius, tenia la concentració més baixa d'AKR1B1 al PS (PS-AKR1B1). A més, no es va observar cap relació entre els nivells de PS-AKR1B1 i els paràmetres de qualitat i funcionalitat dels espermatozoides avaluats a l'arribada al laboratori o després de 72 h de refrigeració. Aquestes troballes van permetre conjecturar que la influència positiva de la PS-AKR1B1 sobre la fertilitat es podria atribuir a la modulació del desenvolupament embrionari i/o del tracte reproductor femení mitjançant la síntesi de determinades prostaglandines, més que als efectes d'aquesta proteïna sobre la fisiologia espermàtica. Finalment, l'últim treball del Capítol 1 pretenia avaluar la relació entre la composició de metabòlits del PS i diversos paràmetres fertilitat *in vivo* en porcí, concretament la taxa de parts, la mida de la ventrada, el nombre de garrins morts per ventrada i la durada de la gestació. Dels 24 metabòlits identificats i quantificats en totes les mostres de PS, es va trobar que 12 d'aquests estaven relacionats amb paràmetres específics de fertilitat *in vivo*. Concretament, aquest estudi va mostrar que: i) la concentració de lactat del PS estava associada amb la taxa de parts; ii) les concentracions de carnitina, hipotaurina, glutamat, sn-glicero-3-fosfocolina i glucosa del PS estaven relacionades amb la mida de la ventrada; iii) les concentracions de citrat, creatina, malonat, fenilalanina i tirosina del PS estaven associades amb el nombre de garrins morts per ventrada; i iv) les concentracions de malonat i fumarat del PS estaven relacionades amb la durada de la gestació. Tot i que els estudis previs suggereixen que la majoria d'aquests metabòlits regulen la fisiologia dels espermatozoides, no s'han investigat els seus efectes sobre el tracte reproductor femení. Per aquest motiu, els estudis posteriors haurien d'escatir els mecanismes a través dels quals aquests metabòlits del PS influeixen en la fertilitat. D'altra banda, el Capítol 2 tenia com a objectiu determinar si els components de

l'esperma, incloent les proteïnes, els metabòlits i la integritat de l'ADN, poden condicionar la fertilitat *in vitro*, concretament la fecundació de l'oòcit i el desenvolupament embrionari pre-implantacional. En particular, el primer treball del Capítol 2 va investigar si l'AKR1B1 dels espermatozoides era capaç de modificar-ne la seva fisiologia i si estava relacionada amb els resultats de fecundació *in vitro* (FIV) a l'espècie porcina. Els resultats van mostrar que els nivells de l'AKR1B1 potencialment inactiva de l'espermatozoide estaven relacionats negativament amb la motilitat espermàtica i els nivells de calci intracel·lular, paràmetres lligats a la capacitació prematura. A més, es va observar que nivells més alts de la forma potencialment inactiva de l'AKR1B1 estaven relacionats amb un menor èxit de la FIV, tant pel que fa a la fecundació d'oòcits com al desenvolupament embrionari. Així doncs, a partir d'aquests resultats es podria suggerir que l'AKR1B1 dels espermatozoides estaria implicada en la regulació de la seva capacitat fecundant. D'altra banda, i atès que el tercer estudi del Capítol 1 va observar que hi havia una associació entre la fertilitat *in vivo* i els metabòlits del PS relacionats amb les rutes energètiques, el segon treball del Capítol 2 va analitzar la relació entre el metabolisme dels espermatozoides i els resultats de FIV en porcí. Les dades van assenyalar que les mostres d'espermatozoides d'alta qualitat estaven associades amb nivells més alts de metabòlits derivats de la glucòlisi, la qual cosa suggereix que l'espermatozoide porcí utilitzaria la glucòlisi com a via catabòlica principal. Curiosament, també es va observar que els espermatozoides que utilitzaven preferentment la glucòlisi estaven associats amb un percentatge més alt d'embrions a dia 6. Aquests resultats van corroborar la hipòtesi que el metabolisme dels espermatozoides podria influir en els resultats de la FIV. La relació de la fertilitat tant amb el metabolisme de l'espermatozoide com amb els nivells d'AKR1B1 dels espermatozoides es podria explicar per la producció de les espècies reactives d'oxigen, que se sap que indueixen dany a l'ADN. Per aquest motiu, l'últim treball del Capítol 2 va investigar si les ruptures de l'ADN dels espermatozoides podrien influir en l'èxit de la FIV

a l'espècie porcina. Les dades van revelar que el dany a l'ADN comprometia el desenvolupament embrionari pre-implantacional, però no la capacitat fecundant dels espermatozoides. De fet, es va veure que tant el nombre total de ruptures de l'ADN com específicament les de doble cadena afectaven negativament al percentatge de blastocists primerencs/blastocists i el percentatge de blastocists que estan eclosionant i que han eclosionat. Addicionalment, també es va veure que el potencial de desenvolupament de les mòrules a dia 6 estava influenciat negativament per les ruptures de doble cadena de l'ADN. Totes aquestes observacions van evidenciar que el dany a l'ADN dels espermatozoides pot retardar el desenvolupament de l'embrió i, fins i tot, promoure l'aturada del desenvolupament en les primeres etapes. En conclusió, els resultats d'aquesta Tesi Doctoral van demostrar que els factors paterns poden influir directament i indirecta l'entorn matern i la capacitat fecundant i posterior desenvolupament de l'embrió. Els estudis posteriors s'haurien de centrar en determinar exhaustivament la comunicació entre mascle-femella-embrió per entendre millor els factors subjacents a l'èxit reproductiu en els mamífers.

Resumen

Durante bastante tiempo, el paradigma dominante en la Biología de la Reproducción ha considerado que el éxito reproductivo estaba determinado únicamente por el genoma de los oocitos y los espermatozoides. Sin embargo, en las últimas décadas, se ha ido evidenciando que hay otros factores, tanto femeninos cuanto masculinos, que también son relevantes. Respecto a estos últimos, se ha propuesto que los factores paternos pueden modular distintos aspectos del proceso reproductivo, incluyendo la fisiología espermática, el entorno materno e, incluso, la salud de la descendencia. Considerando que los mecanismos moleculares que subyacen esta regulación paterna son, en su mayoría, desconocidos, el objetivo de la presente Tesis Doctoral fue determinar el papel que juegan el plasma seminal (PS) y los espermatozoides en el éxito reproductivo, utilizando como modelos animales las especies bovina y porcina. Con este propósito, el Capítulo 1 investigó la capacidad del PS de regular la fertilidad *in vivo*, así como las vías de señalización potencialmente involucradas. En particular, el primer trabajo incluido en el Capítulo 1 examinó si la exposición uterina al PS mejoraba la supervivencia y el desarrollo embrionario en bovino. Los resultados de este estudio demostraron que, aunque el PS no provocaba cambios en el volumen y el peso del cuerpo lúteo ni en la supervivencia embrionaria, los embriones recuperados de los úteros previamente expuestos al PS eran más largos que los del grupo de control y diferían en la expresión de varios genes de desarrollo embrionario, incluidos los siguientes: *CALM1*, *CITED1*, *DLD*, *HNRNPDL*, *PTGS2* y *TGFB3*. Estos datos sugirieron que el PS podría provocar cambios en el ambiente uterino, lo que tendría un impacto positivo en el desarrollo embrionario temprano. Por lo tanto, los siguientes estudios examinaron los supuestos mecanismos moleculares que contribuirían a dicha regulación, incluida la composición de proteínas y metabolitos del PS. En consecuencia, el segundo trabajo del Capítulo 1 tuvo como objetivo aclarar si las proteínas

presentes en el PS podrían modular la fertilidad *in vivo*, utilizando la aldosa reductasa B1 (AKR1B1) como candidata debido a su asociación positiva con mayores tasas de parto en porcino. Los datos de esta investigación mostraron que los primeros 10 mL de la fracción rica en espermatozoides, que contiene aquellas células espermáticas con las mejores características reproductivas, tenían la concentración más baja de AKR1B1 en el PS (PS-AKR1B1). Además, no se observó ninguna relación entre los niveles de PS-AKR1B1 y los parámetros de calidad y funcionalidad del semen evaluados a la llegada de las muestras al laboratorio o después de 72 h de refrigeración. Estos hallazgos permitieron conjeturar que la influencia positiva de la PS-AKR1B1 sobre la fertilidad podría atribuirse a la modulación del desarrollo embrionario y/o del aparato reproductor femenino a través de la síntesis de ciertas prostaglandinas, más que a los efectos directos de dicha proteína sobre la fisiología espermática. Finalmente, el último trabajo del Capítulo 1 pretendía evaluar la posible relación entre la composición de los metabolitos del PS y los resultados de fertilidad *in vivo* en porcino, en términos de tasa de partos, tamaño de la camada, número de lechones muertos por camada y duración de la gestación. De los 24 metabolitos identificados y cuantificados en todas las muestras de PS, se encontró que 12 de estos estaban relacionados con parámetros específicos de fertilidad *in vivo*. Específicamente, este estudio mostró que: i) la concentración de lactato del PS estaba asociada con la tasa de partos; ii) las concentraciones de carnitina, hipotaurina, sn-glicero-3-fosfocolina, glutamato y glucosa del PS estaban relacionadas con el tamaño de la camada; iii) las concentraciones de citrato, creatina, malonato, fenilalanina y tirosina del PS estaban asociadas con el número de lechones muertos por camada; y iv) las concentraciones de malonato y fumarato del SP estaban relacionadas con la duración de la gestación. Aunque la literatura apunta a que la mayoría de estos metabolitos del PS regulan la fisiología de los espermatozoides, no se han investigado sus posibles efectos sobre el tracto reproductivo femenino. Por lo tanto, las próximas

investigaciones deberían analizar a través de qué mecanismos dichos metabolitos influyen en la fertilidad. Por otro lado, el Capítulo 2 buscó determinar en qué medida los componentes del espermatozoide, incluyendo proteínas y metabolitos, así como el daño en el ADN espermático, podrían influir en los resultados de fertilidad *in vitro*, en particular la fecundación de los oocitos y el desarrollo embrionario pre-implantacional. En particular, el primer trabajo del Capítulo 2 exploró si, en porcino, la AKR1B1 de los espermatozoides estaba relacionada con su fisiología y el éxito de la fecundación *in vitro* (FIV). Los resultados mostraron que los niveles de la AKR1B1 potencialmente inactiva presente en los espermatozoides estaban asociados negativamente con la motilidad y los niveles de calcio intracelular, parámetros - ambos - vinculados con la capacitación prematura. Además, los niveles más altos de la forma potencialmente inactiva de la AKR1B1 se relacionaron con un menor éxito de la FIV, tanto en términos de fecundación de los oocitos cuanto de desarrollo embrionario. Basándose en estos datos, por lo tanto, se podría sugerir que la AKR1B1 de los espermatozoides está implicada en la regulación de su capacidad fecundante. Por otra parte, y dada la asociación entre los metabolitos del PS relacionados con la producción de energía y los resultados de fertilidad *in vivo* identificada en el tercer trabajo del Capítulo 1, el segundo estudio del Capítulo 2 analizó la relación entre el metabolismo de los espermatozoides y el éxito de la FIV en la especie porcina. Los resultados mostraron que las muestras de espermatozoides de alta calidad se asociaron con mayores niveles de metabolitos de la glucólisis, lo que sugeriría que el espermatozoide porcino utiliza preferentemente la glucólisis como vía catabólica. Curiosamente, también se observó que los espermatozoides que usaban la glucólisis se asociaban con un mayor porcentaje de embriones en el día 6. Estos resultados respaldaron la hipótesis que el metabolismo de los espermatozoides podría influir en los resultados de la FIV. La influencia observada en el éxito de la FIV tanto del metabolismo de los espermatozoides cuanto de los niveles de

AKR1B1 de aquellos se podría explicar por la producción de especies reactivas de oxígeno, que se sabe que inducen daño en el ADN. El último trabajo del Capítulo 2, por lo tanto, investigó si las rupturas en el ADN de los espermatozoides podrían condicionar los resultados de la FIV en cerdos. Los datos revelaron que el daño en el ADN comprometía el desarrollo embrionario, pero no la capacidad fecundante de los espermatozoides. De hecho, se encontró que el total de roturas y en particular las de doble cadena afectaban negativamente al porcentaje de blastocistos tempranos/blastocistos y el porcentaje de blastocistos que eclosionaban o habían eclosionado. Además, también se vio que el potencial de desarrollo de las mórulas en el día 6 estaba influenciado negativamente por las roturas de doble cadena del ADN espermático. Todos estos hallazgos evidenciaron que el daño en el ADN espermático puede retrasar el desarrollo del embrión e, incluso, promover su detención en etapas tempranas del mismo. En conclusión, los hallazgos de esta Tesis Doctoral indicaron que los factores seminales pueden modular directa e indirectamente el entorno materno y la fecundación y el desarrollo del embrión. Las investigaciones ulteriores deberían centrarse en determinar cómo se produce la interacción macho-hembra-embrión para comprender mejor qué factores subyacen al éxito reproductivo en los mamíferos.

Introduction

1 Semen

Semen is a complex mixture containing a cellular fraction, formed by male gametes called sperm, and a liquid fraction, named seminal plasma (SP), comprised of secretions from the male reproductive tract. The main function of semen has traditionally been associated with the delivery of male gametes into the female reproductive tract to enable the fusion with their female counterparts, the most elementary process of mammalian reproduction (Garner and Hafez, 2000).

1.1. Sperm

Sperm are male gametes, the haploid cells acting as vehicles for transmission of paternal information to the offspring. They are one of the most specialised cells of the mammalian body, adapted to their function and to the changing environments to which they are exposed to throughout their lifespan. The reproductive strategy of each mammalian species is believed to directly shape sperm in terms of physiology and morphology (Garner and Hafez, 2000; **Figure 1**).

1.1.1. Sperm morphology

Sperm have two distinct parts: the head and the tail (**Figure 1**). The **head** covers two relevant structures: the nucleus, which contains the genetic information, and the acrosome. Sperm have a haploid genetic cargo that is considered transcriptionally inactive (Baker and Aitken, 2009). This is because the sperm chromatin is highly condensed due to the presence of protamines, a superfamily of small arginine-rich proteins synthesised in late-stage spermatids (Balhorn, 2007). On the other hand, the acrosome is a double-layered membrane vesicle located at the anterior end of the sperm head. This structure mainly contains hydrolytic enzymes, such as acrosin

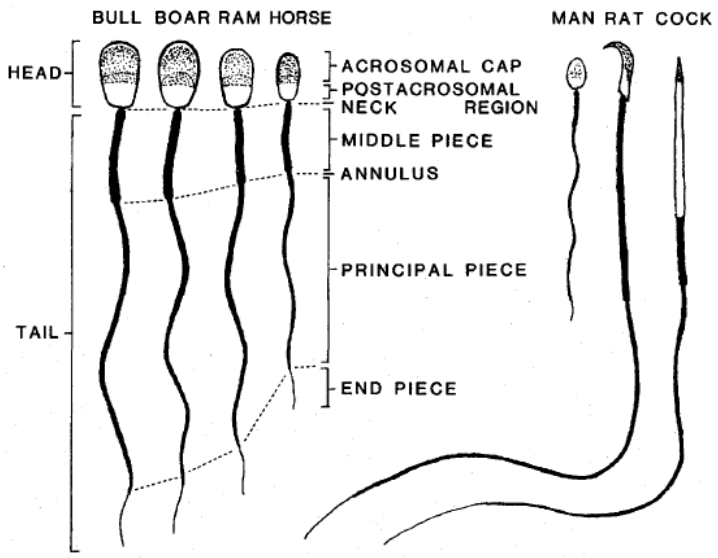

***Figure 1. Inter-species differences of* sperm morphology.** Differences in shape and size of sperm can be observed between vertebrate species. The main morphological structures can also be noted (Garner and Hafez, 2000).

and hyaluronidase, which are required for sperm to pass through oocyte vestments (Khawar et al., 2019). The next structure, the **tail** or **flagellum**, can be also segmented into three regions: the middle piece, principal piece, and end piece. First, the neck connects the region between the head and the tail. This is followed by the middle piece, which is the portion of the tail where mitochondria are located. Sperm mitochondria are singularly arranged in a helical pattern longitudinally to the middle piece of the tail; they produce energy and are involved in capacitation. The principal piece comprehends most of the tail, and has a fibrous sheath that stabilises the cell when the tail contracts during sperm movement. Finally, the end piece is the termination of the fibrous sheath (Garner and Hafez, 2000). Alongside the tail, there is an evolutionarily conserved, specialised subcellular structure known as the axoneme, which provides sperm with motile capacity. This structure begins in the centre of the middle piece and terminates at the end piece. The axoneme is composed of a nine-doublet microtubule encircling two central singlet microtubules (9 + 2 axoneme), which are enclosed by dense fibres that vary along the tail. For instance, while dense fibres

peripherally surround the axoneme in the middle and principal pieces, a central axoneme only covered by plasma membrane can be found at the end of the tail (Garner and Hafez, 2000; Linck et al., 2016).

1.1.2. Originating the male gamete: spermatogenesis and spermiogenesis

The formation of gametes is driven by reductive meiotic divisions, which are highly conserved in the eukaryotic domain. This process is very different between males and females. In the case of the male, the process by which sperm are produced is called **spermatogenesis**.

In the course of male embryogenesis, primordial germ cells (PGCs) colonise the genital ridge to be incorporated into the sex cords, the gonad primordium that later differentiates into the seminiferous tubules. Once in the gonads, PGCs begin to divide to become type A spermatogonia, the stem cells of the seminiferous epithelium morphologically characterised as small cells with an ovoid nucleus. The gamete formation process is stopped until male puberty, when spermatogenesis is initiated. First, spermatogonia divide into another spermatogonia (for self-renewal purposes) and intermediate spermatogonia, which are committed to differentiate into type B spermatogonia, the last cells that undergo mitosis in the germ line. Thus, these type B spermatogonia divide to generate primary spermatocytes, the first cells entering meiosis. When primary spermatocytes complete the first meiotic division, they become secondary spermatocytes, which complete the second meiotic division. The resulting haploid cells are the round spermatids, which undertake a complex differentiation to become spermatozoa (Gilbert, 2000). This cellular specialisation is named **spermiogenesis**, and mainly comprises five events: i) the formation of the acrosome from Golgi apparatus; ii) the growth of the tail from the relocated centriole; iii) the localisation of mitochondria around the middle piece of the tail; iv) the compaction of chromatin into a transcriptionally inactive

genome through histone replacement by protamines; and v) the removal of the remaining cytoplasm and unnecessary organelles (e.g., Golgi apparatus or endoplasmic reticulum) through residual bodies (Goossens and Tournaye, 2017). Next, sperm are successively released into the seminiferous tubule lumen, a process known as **spermiation**. Each of the aforementioned cell types occupies a specific stratum of the seminiferous epithelium, the spermatogonia being in contact with the lamina basalis, and the resulting sperm apically in the seminiferous epithelium and even in the lumen of the tubule.

Upon release from the testis and albeit highly differentiated cells, sperm are unable to move and have no fertilising capacity. To reach their full fertilising potential, sperm must undergo two extra-testicular maturing processes: the first one in the male reproductive tract, known as epididymal maturation; and the second in the female reproductive tract, named capacitation (Gervasi and Visconti, 2017). Both processes are explained in detail later in this Dissertation.

1.1.3. Epididymal maturation

The epididymis is a tubular organ divided in three differentiated anatomical regions: the caput, the corpus and the cauda. These segments vary in terms of gene expression, ion concentration, function and histological appearance. For instance, whereas the thickness of the epididymal epithelium decreases throughout the conduit, the diameter of the lumen increases. In addition, sperm concentration gradually raises along the epididymal transit as a result of water reabsorption (Sullivan and Mieusset, 2016).

Epididymal maturation involves the modification of sperm surface to acquire both flagellar beating and fertilising ability. In this regard, some of the most relevant physiological modifications include changes in

membrane lipid composition (Rejraji et al., 2006), surface glycoproteins (Tulsiani, 2006) and antigen localisation (Belmonte et al., 2000), remodelling of raft membrane microdomains (Girouard et al., 2011), cytoplasmic droplet loss (Gervasi and Visconti, 2017) and acquisition of forward motility (Vadnais et al., 2013), among others. In addition, the cauda epididymis also serves as a reservoir until ejaculation occurs (Sullivan and Mieusset, 2016).

The exact underlying molecular mechanisms that regulate sperm epididymal maturation are yet to be uncovered, but epididymosomes, the extracellular vesicles (EVs) secreted by epididymal epithelial cells, appear to play a critical role. Particularly, epididymal cells seem to be able to modify immature sperm through the secretion of epididymosomes, which have been reported to be able to transfer their bioactive cargo (Suryawanshi et al., 2012; Caballero et al., 2013). Epididymosomes cargo has been reported to include several proteins directly involved in fertilisation. Some examples are: zona pellucida (ZP) sperm binding proteins and A Disintegrin And Metalloprotease (ADAM) superfamily proteins, which are involved in oocyte recognition and adhesion (Nixon et al., 2019); and macrophage migration inhibitory factor (MIF), which is a crucial factor for sperm motility (Eickhoff et al., 2004).

Ejaculates are a mixed heterogeneous population of normal and abnormal sperm. Morphologically aberrant sperm can originate from spermatogenesis (primary alterations), epididymal maturation (secondary alterations), or improper semen handling (tertiary alterations). From these, secondary alterations, which mainly include proximal and distal cytoplasmic droplets, are the most common (Bonet et al., 2012). Primary and secondary alterations usually reflect impaired testicular and epididymal function, and have been largely used to predict the fertility potential of semen samples (Ombelet et al., 1995).

1.2. Seminal plasma

The SP is the liquid fraction of semen, composed of secretions from the epididymis and accessory glands that are mixed upon ejaculation. The traditional role attributed to SP has been to carry, protect and nourish sperm during and after ejaculation (Garner and Hafez, 2000). Yet, in the last decades, the functional significance of SP has been questioned. Indeed, components of SP have been proposed to modulate sperm physiology in a series of crucial events, including the transport of the male gamete along the female reproductive tract, the formation of the oviductal reservoir, sperm capacitation and even fertilisation (Rodriguez-Martinez et al., 2021). In addition, SP also seems to be able to regulate sperm energy production through its molecular composition in terms of proteins and metabolites (Rodriguez-Martinez et al., 2011; Bromfield, 2016). Remarkably, in recent years, SP has been suggested to affect the reproductive success beyond its effects on sperm. In this regard, it has been reported that SP is able to modulate the immune environment in the female genital tract, thus facilitating conception and pregnancy in mammals (Rodriguez-Martinez et al., 2021; Ahmadi et al., 2022). Interestingly, the relevance of SP in the process of fertilisation seems to be tightly associated to the reproductive strategy of each species, being more significant in species with vaginal deposition (i.e., humans, small ruminants or cattle) than in those with an uterine one (i.e., porcine and equine; Schjenken and Robertson, 2014).

1.2.1. Origin and composition

The SP is a heterogeneous complex fluid composed of inorganic ions, sugars, salts, lipids and, mainly, proteins from secretions of the epididymis and accessory sex glands, which basically consist of seminal vesicles, prostate and bulbourethral glands.

From an interspecies perspective, there are variations in the size and/or presence of accessory sex glands. For example, while boars have prostate, seminal vesicles and bulbourethral glands, rams and bulls present a relatively small and disseminated prostate gland, camelids lack seminal vesicles and dogs only have prostate, lacking from the other accessory glands (Druart et al., 2013). Not only do these anatomical disparities imply differences in the ejaculate volume, but also in the SP composition. In effect, variations in the SP proteome have been observed between species (Druart and de Graaf, 2018). This has been inferred to occur in response to reproductive strategy requirements.

On the other hand, in species where the ejaculate is sequentially emitted in fractions, such as dogs, horses, pigs and humans (Rodriguez-Martinez et al., 2021), intra-ejaculate differences in SP molecular composition and volume have been noticed. In more detail, and using the pig as an example, three distinct ejaculate fractions can be identified: i) the pre-ejaculate fraction, emitted first and composed of urethral and/or bulbourethral glands secretions, which confer its watery aspect; ii) the sperm rich fraction (SRF), which contains most of the ejaculated sperm together with epididymal and prostate secretions, with little contribution from seminal vesicles; iii) the post-SRF, which is mainly composed by seminal vesicles secretions and is poor in sperm; and iv) the gel-rich fraction at the end of ejaculation, composed by coagulating bulbourethral glands secretions (Saravia et al., 2009; Rodriguez-Martinez et al., 2011). The pig SRF can be further divided into two distinct portions: the first 10 mL of the SRF (SRF-P1) and the rest of the SRF (SRF-P2). Considering the large volume of the ejaculate, most studies characterising the composition of SP and the features of sperm cells in the separate ejaculate fractions and SRF-portions have been mainly carried out in pigs. In the case of sperm functionality, differences between ejaculate fractions and SRF-portions have also been reported. Accordingly, the sperm contained in the SRF-P1 seem to hold the best traits, specifically exhibiting the highest motility, viability (Rodríguez-

Martínez et al., 2005) and resilience to liquid-storage (Sellés et al., 2001) and cryopreservation (Peña et al., 2003; Saravia et al., 2009; Alkmin et al., 2014; Li et al., 2018). The variability in the molecular composition of SP within the ejaculate has been proposed to explain the differences in sperm performance between ejaculate fractions and SRF-portions (Alkmin et al., 2014; Barranco et al., 2015; Li et al., 2018). In fact, the proteomic (Perez-Patiño et al., 2016) and metabolomic (Mateo-Otero et al., 2020) profiles of pig SP have been found to vary among the distinct ejaculate fractions and SRF-portions. It cannot be discarded, however, that these differences are also due to the inherent characteristics of the sperm contained in each fraction, as their miRNA (Martinez et al., 2022) and protein (Pérez-Patino et al., 2019) profiles have also been found to vary.

Collectively, the molecular composition of SP appears to be very relevant for the modulation of mammalian sperm physiology. For this reason, the first Chapter of the present Dissertation addressed whether the modulation of the female reproductive tract and sperm by SP ends up influencing sperm fertilising ability, and the subsequent embryo development.

2 Sperm within the female reproductive environment

2.1. Capacitation and Acrosome reaction

Upon ejaculation, sperm surrounded by the epididymal fluid enter the vas deferens, where they are mixed with sex accessory glands secretions. This mixture forms the final ejaculate, which is first ejected through the urethral meatus and later deposited within the female reproductive tract (Alwaal et al., 2015).

Once deposited into the female reproductive tract, mammalian sperm must undergo several biochemical and physiological modifications to become fertilising competent. The changes, which prepare sperm for successful binding to oocyte ZP and fertilisation, are collectively called capacitation. For instance, capacitated sperm are more able to chemotactically move towards the oocyte through progesterone (P4) gradients (Teves et al., 2009; Gatica et al., 2013). Capacitation has been proposed to be divided into slow and fast events (Visconti, 2009; Yeste, 2013a), as detailed below.

The **fast events** of capacitation begin with the activation of sperm motility. This starts as soon as sperm encounter an isotonic media like SP, which contains bicarbonate (HCO_3^-) and calcium (Ca^{2+}) that stimulate a unique, sperm-specific adenylyl cyclase called Soluble Adenylyl Cyclase (SACY). The production of cyclic adenosine monophosphate (cAMP) leads to the activation of Protein Kinase A (PKA), which induces the phosphorylation of proteins that subsequently trigger several signalling pathways (Salicioni et al., 2007; Ickowicz et al., 2012).

On the other hand, the **slow events** of capacitation are initiated by the efflux of cholesterol and the reorganisation of plasma membrane architecture, which results in an increase of membrane fluidity and permeability (Flesch et al., 2001). Next, the raise in the intracellular levels of HCO_3^- and Ca^{2+} ultimately triggers SACY and, in turn, PKA, that phosphorylates its substrates, which mainly result in: i) changes of the sperm motility pattern into a faster and straight movement, known as hyperactivation; ii) increase in protein tyrosine phosphorylation; iii) hyperpolarisation of sperm plasma membrane as a result of changes in the activity of ion-selective channels and transporters; iv) polymerisation of globular (G)-actin to filamentous (F)-actin; and iv) reorganisation of membrane lipids and proteins of plasma and outer acrosome membranes to become fusogenic for posterior acrosome reaction (Breitbart et al., 2005;

Salicioni et al., 2007; Visconti, 2009; Tsai et al., 2010; Yeste, 2013a; Jin and Yang, 2017).

At the end of capacitation, the outer acrosomal membrane and the overlying plasma membrane fuse at multiple points. In response to physiological or pharmacological stimuli, the acrosomal content is released in a process known as the **acrosome reaction**. In mammals, where and how acrosome reaction is triggered is still under debate. For a long time, while some authors considered that sperm initiate acrosome reaction while advancing through the cumulus cells mass surrounding the oocyte (Yanagimachi and Phillips, 1984; Gahlay et al., 2010; Jin et al., 2011), others advised that the acrosome reaction is initiated when sperm bind ZP proteins (Florman and Storey, 1982; Cherr et al., 1986; Schroer et al., 2000). In the last decade, however, a third model has been hypothesised, purporting that the acrosome reaction is induced by factors such as the P4 present in the oviduct and released by cumulus cells and, even, the follicular fluid (Buffone et al., 2014). The acrosome reaction, nevertheless, is relevant for fertilisation not only because of the release of lytic enzymes able to digest and dissociate cumulus–oocyte complex (COC) glycoproteins, thus allowing sperm penetration, but also because of the exposure of the inner acrosome membrane, which is relevant for sperm binding to oolemma prior to gamete fusion (Hirohashi and Yanagimachi, 2018).

Capacitation and acrosome reaction are known to be highly regulated processes by both female and male factors. The molecular basis of this regulation, notwithstanding, is poorly understood. Regarding the female side, one of the most well-described regulating factors playing an important role in the process is P4, which is usually secreted by cumulus and granulosa cells during ovulation and by the corpus luteum (CL) during the post-ovulatory phase (López-Torres and Chirinos, 2017). This hormone has been reported to increase hyperactivation, induce Ca^{2+} influx and trigger the acrosome reaction (Jin and Yang, 2017). On the male side, many efforts

have been made to describe how SP molecules regulate capacitation events. In general, SP is assumed to contain decapacitation factors that prevent premature sperm capacitation before reaching the oocyte (Aitken and Nixon, 2013). One example are porcine seminal plasma (PSP) proteins, which are able to maintain pig sperm survival and delay capacitation (Caballero et al., 2009). Yet, in the case of bovine seminal plasma (BSP) proteins, which coat sperm surface via binding to choline phospholipids, the control of capacitation is more complex. For instance, BSP proteins have been observed to differently regulate capacitation depending on the presence of other agents (Gwathmey et al., 2006). In particular, while BSP proteins are able to stabilise sperm membrane and hinder capacitation from being elicited (Manjunath and Thérien, 2002), they are able to indirectly induce sperm capacitation in the presence of heparin and high-density lipoproteins (Parrish, 2014). Specifically, heparin and high-density lipoproteins have been reported to induce cholesterol efflux resulting in plasma membrane reorganisation and downstream signalling towards a capacitation status in epididymal sperm (Manjunath and Thérien, 2002). In addition, seminal EVs have also been suggested to act both promoting (Siciliano et al., 2008; Murdica et al., 2019) and delaying (Pons-Rejraji et al., 2011; Piehl et al., 2013; Du et al., 2016) sperm capacitation. All these findings evidence that the exact molecular mechanisms by which capacitating events are regulated remain unclear. Bearing in mind the relevance of capacitation in the reproductive process, research on this topic is still needed to provide a complete picture of the mechanisms involved in the acquisition of sperm fertilising ability, which could be targeted to improve poor fertility outcomes.

2.2. Oocyte fertilisation

Upon ejaculation, millions of sperm are deposited in the female reproductive tract. In spite of this, only a few reach the oocyte in the ampulla

of the oviduct to initiate fertilisation. Fertilisation is a key step in sexual reproduction, and encompasses two sequential steps: sperm recognition of the oocyte and gamete fusion to form a zygote (**Figure 2**).

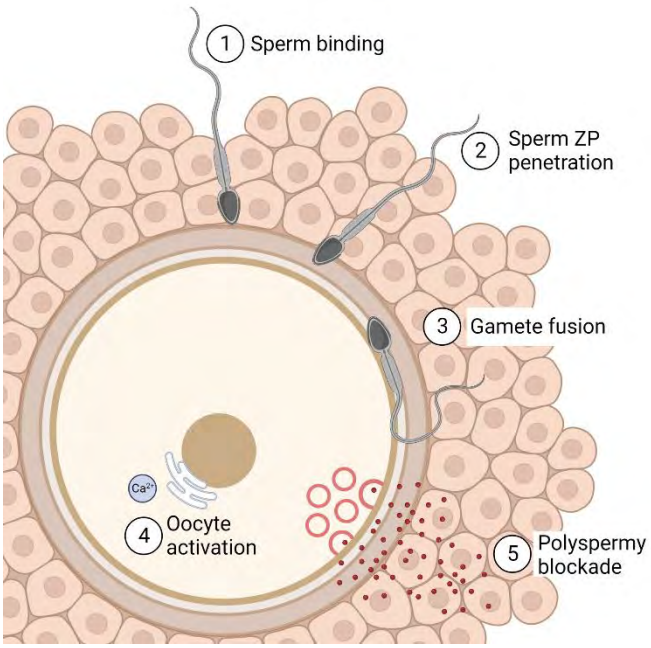

Figure 2. **Mammalian fertilisation.** After crossing cumulus cells mass, sperm bind (1) and penetrate oocyte zona pellucida (2) to adhere oocyte plasma membrane (oolemma). Next, gametes fuse (3) and the oocyte is activated (4), resulting in the release of the cortical granules to prevent polyspermy (5). Created with BioRender.

As explained above, a prerequisite for a sperm cell to fertilise an oocyte is capacitation. Only capacitated sperm are able to penetrate cumulus cells through the disaggregation of this cellular mass, traditionally thought to occur by means of hyperactivated motility and acrosome reaction. Then, sperm reach and penetrate the oocyte ZP. Heterospecific sperm penetration of ZP-free oocytes has widely demonstrated the key role of ZP during fertilisation (Zhao et al., 2002). The exact molecular mechanisms underlying **sperm binding to oocyte ZP** are, however, still under debate. One of the most widely accepted models is that ZP glycoproteins, including ZP1, ZP2, ZP3 and ZP4, are recognised by sperm receptors. It is worth highlighting that differences in the content of ZP isoforms exist between species, as neither ZP1 is expressed in cattle or pigs nor is ZP4

found in mouse (Yeste, 2013b; Tumova et al., 2021). Similarly, while binding receptors, such as galactosyltransferase, proacrosin/acrosin, zonadhesin and arylsulphatase A, seem to be conserved across species, species-specific sperm-ZP binding proteins like spermadhesins and zona receptor in pig and human sperm, respectively, have also been identified (Tumova et al., 2021). Either way, it remains unaddressed whether one or more sperm receptors are required for this process. In addition, the debate continues regarding acrosome integrity during sperm penetration through COCs layers and, thus, about the specific role of the acrosomal cargo during oocyte fertilisation (Bhakta et al., 2019). For this reason, as briefly mentioned before, it is unclear whether the sperm capacity to bind ZP is only restricted to acrosome-reacted sperm or rather rely upon the specific mechanisms by which sperm are able to cross ZP. Research on the mechanisms inducing the acrosome reaction and on the molecular mediators of sperm-oocyte binding is much required for a clear understanding of gamete recognition in mammalian species.

In addition, after sperm binding, sperm need to undergo **ZP penetration** before encountering the oocyte. This process has been demonstrated to occur due to both sperm flagellar forces and acrosome reaction (Kozlovsky and Gefen, 2013). Notwithstanding how the acrosome reaction is initiated is still under debate, it is widely accepted that proteins contained in that acrosome are required for ZP penetration. For example, acrosin (Hirose et al., 2020) and 26S proteasome (Zimmerman et al., 2011) have been demonstrated to be indispensable for zona penetration in mammals.

Gamete fusion is the process through which the oocyte and the sperm plasma membranes adhere and fuse. Although many efforts have been made towards the identification of the mechanisms governing gamete adhesion, only a small list of molecules have been hitherto identified to be implicated in this process: i) from the sperm side: Izumo sperm-oocyte

fusion 1 (IZUMO1) (Inoue et al., 2005), transmembrane protein 95 (TMEM95) (Pausch et al., 2014) and sperm acrosome membrane-associated protein 6 (SPACA6) (Lorenzetti et al., 2014); and ii) from the oocyte side: folate receptor 4 (FOLR4 or JUNO) (Bianchi et al., 2014) and tetraspanins CD9 (Le Naour et al., 2000) and CD81 (Rubinstein et al., 2006). From those, the most well-described mechanism involves IZUMO1-JUNO interaction, which seems to be conserved across mammalian species (Bianchi et al., 2014; Grayson, 2015). Briefly, the local clustering of JUNO in the oolemma (probably mediated by tetraspanin CD9) allows the attachment to IZUMO1 (Chalbi et al., 2014), thus leading to gamete binding and subsequent fusion of plasma membranes. The most accepted model for gamete fusion involves tetraspanins, as it seems that sperm tetraspanins CD9 and CD81 interact with their oocyte counterparts, which include CD9P-1, α6β1 integrin and IGSF8 (Bhakta et al., 2019). Yet, the participation of other mechanisms cannot be discarded; thus, the exact molecular mechanisms still need to be elucidated.

The **activation of the oocyte** is usually considered the first vital step of embryogenesis (Kashir et al., 2012). In mammals, this activation is triggered by repeated calcium oscillations induced by sperm (Kline and Kline, 1992), which mediate the release of that calcium from the endoplasmic reticulum of the oocyte (Kashir et al., 2012). In fact, Saunders et al. (2002) found a PI-specific phospholipase in sperm, the 1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Zeta-1 (PLCζ-1), able to induce calcium oscillations in oocytes (Saunders et al., 2002). In particular, injection of *PLCZ1*-cRNA or the recombinant protein into mouse oocytes was found to induce calcium oscillations required for embryo development progression (Saunders et al., 2002; Kouchi et al., 2005). This enzyme is able to hydrolyse Phosphotidylinositol 4,5-Bisphosphate (PIP_2) into Inositol Triphosphate (IP_3) and Diacylglycerol (DAG). Consequently, DAG activates Protein Kinase C (PKC), which phosphorylates several proteins, and IP_3 interacts with its receptor and mobilises the calcium from

the endoplasmic reticulum (Malcuit et al., 2006). The frequency and amplitude of these calcium waves is highly variable across species. For instance, whereas mice oocytes have low frequency calcium spikes every 10 minutes, in humans, pigs and cattle oocytes, this occurs every 30 to 60 minutes (Zafar et al., 2021). In any case, these calcium oscillations allow the progression of the cell cycle, which entails extrusion of second polar body and the exocytosis of oocyte cortical granules (Kashir et al., 2022).

Finally, the fertilisation process in mammals is usually assumed to terminate with oocyte **polyspermy blockade**. The mechanism by which mammalian oocytes prevent polyspermy involve the exocytosis of cortical granules promptly after fertilisation. The release of the content of these granules in response to calcium oscillations prevents sperm binding due to proteinases that modify proteins to harden ZP (Liu, 2011), and zinc that disrupts both the ZP structure (Que et al., 2014) and sperm motility (Tokuhiro and Dean, 2018). This is followed by oocyte membrane blocking to polyspermy, which occurs as a result of the modification of the oolemma molecular composition after sperm membrane fusion and the increased cytosolic calcium levels (Evans, 2020). Although this process is poorly understood, it seems that this blockade has different efficiency across mammals, which has been proposed to explain the varying incidence of polyspermy between species (Gardner and Evans, 2005).

3 Early embryo development

3.1. Preimplantation embryo stages

The preimplantation embryo is the earliest autonomous form of conceptus development and begins right after fertilisation. It is first sustained by maternal transcripts present in the oocyte and, after embryo genome activation, preimplantation embryos rely on their self-produced proteins and other molecules (Duranthon et al., 2008). Yet, a communication

between the embryo and the maternal environment exists: while oviductal and uterine factors are able to influence key embryo processes such as embryo genome activation or implantation, embryo factors are also able to modulate female reproductive tract physiology (Kölle et al., 2020).

Preimplantation development of mammalian embryos is usually divided into three distinct stages: cell cleavage, formation of morula, and formation of blastocyst. During this free-living period, the mammalian conceptus passes through the oviduct reaching the uterus at the blastocyst stage, where the embryo attaches and implants. At this point, the maternal-foetal interactions required to support embryogenesis are initiated and post-implantation embryo development begins (Watson and Barcroft, 2001).

Initially, the fertilised oocyte, also known as **zygote**, undergoes sequential subdivision into smaller cells restricted in size by the ZP; this process is acknowledged as **cell cleavage**. Zygotes and two-cell embryos are considered totipotent, meaning that cells have the potency to give rise to embryonic and extraembryonic linages. Remarkably, progenitor cells formed from these cleavages become progressively restricted in terms of developmental potency (Suwińska, 2012). Many efforts have been made towards the identification of the mechanisms underpinning this pre-patterning, describing elements such as cell polarity, cell position, mechanical forces and metabolism as crucial (Yao et al., 2019).

Cellular subdivisions result in a 16-cell embryo that undergoes a process called compaction. Compacting embryos result from the increase of intercellular contact that mask distinct individual cell boundaries to form a uniform cell mass known as **morula** (Watson and Barcroft, 2001). At this point, the cell fate of each cell (also called blastomeres) begins to segregate into inner cell mass (ICM) and trophectoderm (TE) (Johnson and Ziomek, 1981). Compacted morula then begins a cavitation process to form the blastocoel. This process is mediated by tight and adherens junctions

established between cells to set a permeability barrier (Kim and Bedzhov, 2022). This is followed by an influx of water from the external environment promoted by aquaporins and ion channels, which gradually increases the inner pressure. Blastocoel formation occurs through the disruption of intercellular junctions at basolateral domains, a process also known as hydraulic fracking (Dumortier et al., 2019). The emergence of this aqueous cavity disrupts the radial symmetry of the compacted morula, establishing embryonic (i.e. ICM cells) and abembryonic (i.e. TE cells) poles still surrounded by the ZP; this corresponds to the **blastocyst** stage. After multiple cell divisions to allow blastocyst expansion, embryos undergo a hatching process crucial for implantation. Although the specific mechanisms inherent to this process are not fully described, blastocyst hatching appears to be highly dependent on ion transport rate, which increases blastocoel water volume and thus the embryo size, and reduces the ZP thickness (Leonavicius et al., 2018).

Whilst early embryo development is significantly conserved across species, it is highly variable in terms of embryo divisions, genome activation timing and metabolic requirements (Vajta et al., 2010). Another distinctive feature is that, unlike humans and mouse, the hatched blastocysts of ungulates do not attach to the uterus during the peri-implantation period and concomitant gastrulation, allowing the extraembryonic tissues transition from a sphere to ovoid, tubule and filament shapes. Trophoblast elongation confers a larger placental area to enable a higher nutrient exchange (Bolmberg et al., 2008). Interestingly, the attempts to induce *in vitro* elongation of ungulates conceptus have been unsuccessful, pointing to a necessary maternal-embryo crosstalk during this process.

3.2. Zygote reprograming and embryo genome activation

Promptly after fertilisation, maternal and paternal DNA need to reprogram in order for the resulting zygote to hold totipotent potential. The ability of maternal products to drive this reprogramming was demonstrated 60 years ago through cloning experiments (Gurdon, 1962). Specifically, the transference of somatic cell nuclei into enucleated oocytes was found to be able to reprogram tissue-specific DNA signatures and give rise to embryos. The underlying molecular mechanisms were further investigated, finding that reprogramming of the zygote genome mainly involves both the exchange of protamines for histones in the paternal chromatin and significant epigenetic modifications of the zygote genome (Duranthon et al., 2008; Fraser and Lin, 2016). In addition, maternal content was also described as being capable of modulating multiple processes besides genome reprogramming, including pronuclear formation and fusion, the first cell division, embryo genome activation and the onset of embryogenesis (Li et al., 2013).

If embryos continue to develop beyond the zygote stage, transcriptional control is switched from the oocyte to the zygote, involving the degradation of maternal products as well as embryo genome activation (**Figure 3**). Several authors have proposed that genome activation should be understood as progressive over a period of time rather than a single event in a specific moment (Schulz and Harrison, 2019). Most models propose that zygote genome activation occurs in two transcriptional waves: a minor wave happening during early cleavage divisions, and a major wave that coincides with the first division-cycle pause in some species (Tadros and Lipshitz, 2009).

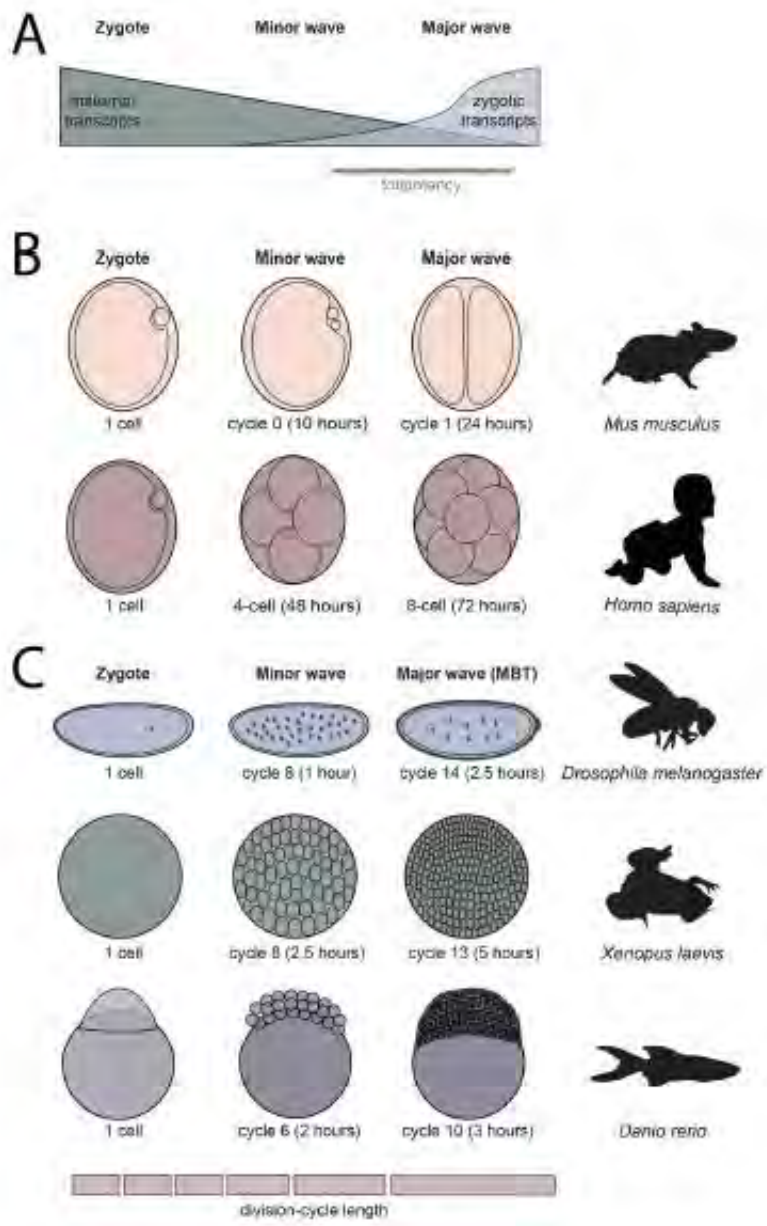

Figure 3. **Embryo genome activation in different species.** (A) Progressive transition from maternal to embryo transcripts. (B, C) Key stages of embryo genome activation in five model species. (Schulz and Harrison, 2019)

3.3. Impaired embryo cleavage and preimplantation embryo arrest

Developmental arrest consists in the permanent halt of mitosis in early embryos, which often takes place before the morula stage is reached. The incidence of this phenomenon in *in vitro* produced mammalian embryos is very high, as it is estimated that fewer than 50% of all embryos reach the blastocyst stage (Paonessa et al., 2021; Yang et al., 2022).

Embryo arrest has been conjectured to prevent further development of low-quality or abnormal/damaged embryos. Thus, permanent embryo arrest is believed to happen as the result of a checkpoint mechanism that evaluates the potential of embryos to develop into healthy individuals (Betts and Madan, 2008). Hence, if a major abnormality is detected in cell cycle checkpoints, the cell does not progress to mitosis, which is usually translated into a delay in the divisions to allow its repair. In fact, this is often reflected on a lower-than-expected number of cells in embryos for a given stage. If the existing problem cannot be resolved or is extensive to all embryo cells, the cell cycle ceases and embryo development is arrested. Curiously, this mechanism delaying development and heading to cell cycle arrest is particular of very early embryos as, after blastocyst formation, the preferred error-solving mechanism is cell apoptosis. This could be related to the high division rate of early embryos (Heyer et al., 2000).

Although several mechanisms have been linked to early embryo arrest, the extent of the negative impact of each of these mechanisms on embryo development remains unclear. Some of the proposed causes for embryo arrest in mammals include: i) failed embryo genome activation (Vera-Rodriguez et al., 2015); ii) delayed degradation of maternal transcripts (Sha et al., 2020); iii) high levels of reactive oxygen species (ROS) (Kawamura et al., 2010; Rocha-Frigoni et al., 2015); iv) aneuploidies (Almeida and Bolton, 1998); v) altered regulation of metabolic pathways (Yang et al., 2022); and vi) DNA damage beyond repair (Simon et al., 2017).

Sperm-derived factors have been shown to have a direct effect on early embryo divisions (Vallet-Buisan et al., 2023). Interestingly, Tesarik (2005) proposed that the impact of these factors could be divided into early and late paternal effects. Early paternal effects would coincide with minor embryo gene transcription activity, at very early developmental stages. These alterations in embryo development would be caused by abnormalities in the sperm centriole and oocyte activating factors. The late paternal effect would occur in cleaving embryos undergoing embryo genome activation, and would be related to sperm DNA damage (Tesarik, 2005). Research has further validated this hypothesis, as paternal factors have been found to condition embryo development from zygote and beyond. Interestingly, it seems that not only DNA fragmentation but also other genetic defects, such as chromatin organisation or epigenetic modifications, could restrict embryo development (Colaco and Sakkas, 2018). Yet, although most efforts have been intended to identify the genetic factors affecting embryo development, the contribution of other sperm components present in the cell cytoplasm or plasma membrane cannot be excluded. Remarkably, the extent of the influence of paternal factors on early embryo development was investigated further in this Dissertation.

4 Dialogue between semen and the female reproductive system

4.1. Hormonal control of the female reproductive system: oestrus and menstrual cycles

Preimplantation development in mammalian species not only includes the continuous division of the developing embryo, but also the preparation of the uterus for its implantation. Indeed, the window of uterine receptivity for

embryos is highly timed by hormonal regulation involving oestrogen (E2) and P4.

Although the general hormonal regulation is common among mammals, there are differences between non-primates and primates. Whereas non-primates (including cattle, pigs, sheep, rodents and horses, amongst others) have a cyclic appearance of behavioural sexual activity with endometrium absorption when no embryo is implanted, named oestrous cycle, primates exhibit a regular endometrium shedding at the end of their cycle when no embryo implantation occurs, called menstrual cycle. In addition, the duration of oestrous/menstrual cycle is highly variable, ranging from 4 to 5 days in rodents (Ajayi and Akhigbe, 2020), 18 to 24 days in pigs (Soede et al., 2011), 21 days in cows (Larson and Ball, 1992) and 28 days in humans (Sherman and Korenman, 1975). Moreover, there are differences in the phases of the hormonal cycle between primates and non-primates. First, while in non-primates, the oestrus cycle begins and ends at the oestrus phase (also known as heat) when ovulation occurs, in primates, the menstrual cycle begins and ends with menstruation, and ovulation occurs at mid-cycle. Additionally, whereas the oestrous cycle has four phases, namely proestrus, oestrus, metoestrus and dioestrus, the menstrual cycle is usually divided into proliferative (or follicular) and secretory (luteal) phases. In fact, the follicular phase is usually assumed to be comparable to proestrus and oestrus phases, and the secretory phase to metoestrus and dioestrus (Radi et al., 2009).

In general, mammalian reproduction in females is regulated by the hypothalamus-pituitary-gonad (HPG) axis. The hypothalamus releases Gonadotrophin Releasing Hormone (GnRH), which acts on the anterior pituitary to induce the secretion of Follicle Stimulating (FSH) and Luteinising Hormones (LH). The main function of FSH is to stimulate the development and growth of ovarian follicles. Growing follicles secrete increasing concentrations of E2, leading to a GnRH surge that induces a peak of LH

secretion, triggering ovulation in the dominant follicle (Ball and Peters, 2004). The ovulated oocyte is collected by the oviductal fimbria and transported to the ampulla, where it may be fertilised. Next, the remaining follicular cells transform into luteal cells under the influence of LH to form the CL. The CL is a transient endocrine gland whose main function is the secretion of P4, which prepares the uterus for a possible pregnancy and also exerts a negative feedback on the hypothalamus, and in turn on FSH and LH secretion, to avoid further ovulations (Bosch et al., 2021).

In species with oestrous cycle, in absence of an embryo, the uterus begins to secrete prostaglandin F2α (PGF2α) in response to ovarian E2. PGF2α has a luteolytic effect (regression of the CL), which results in a drop of P4 secretion that leads to the removal of the negative feedback on the hypothalamus and pituitary. As a result, GnRH is secreted again at levels that induce FSH release. Thus, a new follicular wave and dominant follicle recruitment can take place, allowing a novel ovulation to occur (Hansel and Convey, 1983). In species with menstrual cycle, the demise of CL at late luteal phase reduces the levels of E2 and P4, which removes GnRH negative feedback, initiating a new cycle (Bosch et al., 2021). Interestingly, the exact mechanisms of luteolysis in those species are currently unknown, as no luteolysis signals from the uterus have been identified in primates (Bogan et al., 2008).

On the other hand, when the female becomes pregnant, the embryo releases factors able to block a new ovarian cycle (Banerjee and Fazleabas, 2010). These factors are not common between species; for instance, while primate TE cells produce chorionic gonadotropin (CG) to prevent luteolysis, in ruminants, the interferon τ (IFN-T) secreted by the TE seems to have a comparable effect (Bai et al., 2012). These molecules are able to prevent CL luteolysis, which permits the continuous secretion of P4 to keep elevated levels. The high P4 levels are thus able to inhibit the HPG axis to prevent a new cycle in order to support pregnancy. This evidences the relevance of a

correct early crosstalk between the conceptus and the maternal system for a successful pregnancy.

4.2. Modulation of the maternal environment by semen

The modulation of the female reproductive tract has been described to go beyond hormonal self-regulation. As briefly mentioned above, a significant number of studies in the last two decades have pointed out semen as a key regulator of the maternal environment, particularly promoting gestation in a wide range of mammalian species (Schjenken and Robertson, 2014). Accordingly, the signalling function of semen seems to be highly conserved through evolution, suggesting its biological value not only for offspring health but also for species progression (Mcgraw et al., 2015).

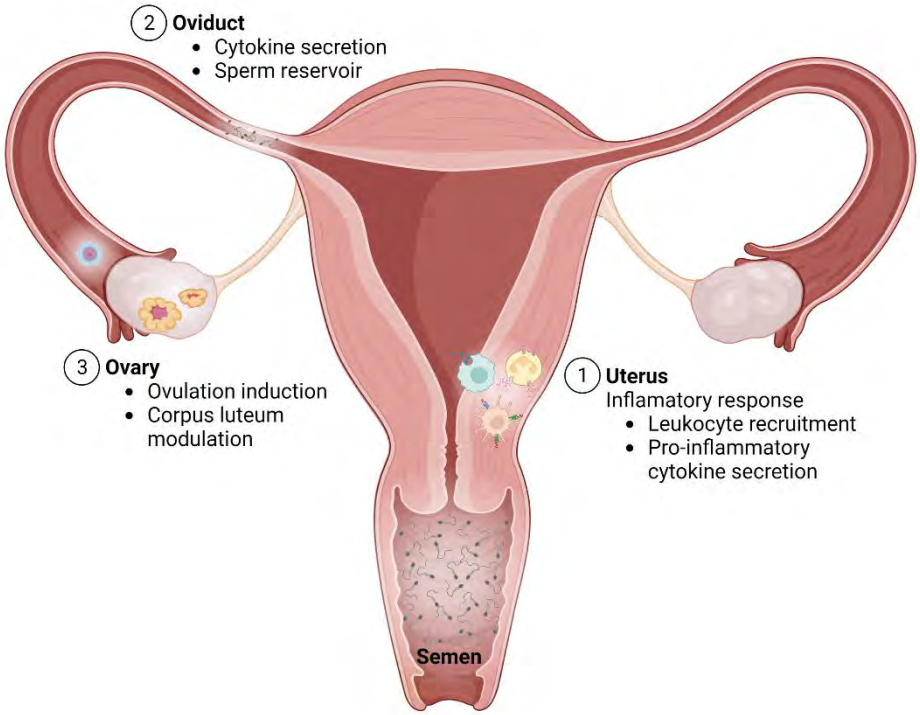

Figure 4. **Female response to seminal components.** Schematic representation of the main regions of the female reproductive tract in which seminal components might be able to act to promote successful pregnancy. Created with BioRender.

Since this research field is still in expansion, the exact extent to which semen is able to modulate pregnancy success remains unknown. Yet, it seems that both SP and sperm can interact with the female reproductive tract at several levels (**Figure 4**). Thus, considering the social, environmental and economic impact of reproduction, a better understanding of how reproductive events are regulated is crucial for an appropriate management in both humans and farm animals (Schjenken and Robertson, 2020). The state-of-the-art is reviewed hereunder.

4.2.1. Involvement of seminal factors in the uterine inflammatory response

Research in this area can be traced back to experiments carried out in rodents, where the removal of accessory glands, particularly seminal vesicles, was found to reduce fertility and increase post-implantation foetal losses in rats (Queen et al., 1981), mice (Pang et al., 1979; Peitz and Olds-Clarke, 1986) and hamsters (O et al., 1988). These studies conjectured the relevance of SP for the reproductive success, opening a wide range of hypotheses about the specific effect of this fluid on the female reproductive tract. Subsequent investigations reported the secretion of pro-inflammatory cytokines in rat and mouse uterus in response to mating, in particular tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6, colony-stimulating factor (CSF)-1 and CSF-2 (Robertson and Seamark, 1990; Robertson et al., 1992; Sanford et al., 1992). In addition, studies in rodents described a recruitment of specific leukocytes such as neutrophils, macrophages and dendritic cells to the endometrium after mating (Robertson et al., 1996; Tremellen et al., 1998). In the following years, similar findings were observed in humans. In particular, cervical biopsies showed an induction of CSF-2, IL-6, IL-8 and IL-1α and leukocyte infiltration in response to seminal fluid, which was not elicited during intercourse using condoms (Sharkey et al., 2012b, 2012a). Certainly, *in vitro* studies confirmed that SP was the main

responsible of triggering such an inflammatory response, as endometrial epithelial and stromal cells cultured with SP presented an increased secretion of pro-inflammatory and chemotactic cytokines (Chen et al., 2014). This post-mating leucocytosis response was found to be conserved across domestic species, including cattle (Ibrahim et al., 2019), horses (Troedsson et al., 2001), sheep (Scott et al., 2006) and pigs (O'Leary et al., 2006). All these findings have placed SP in the spotlight as an essential actor in the regulation of the immune environment in the female reproductive tract. Besides, sperm have also been shown to modulate female leukocyte recruitment in several mammalian species (Schjenken and Robertson, 2020). In summary, after many research efforts, seminal factors have been demonstrated to trigger a classic inflammatory reaction, with an initial surge of pro-inflammatory cytokines and an extensive leukocyte infiltration. Many hypotheses briefly depicted herein about the relevance and role of this immune response for the reproductive success have been proposed.

Initially, the **neutrophil recruitment** happening immediately after coitus was thought to mainly occur for microbial clearance purposes (Schjenken and Robertson, 2014). Yet, a second key role for these cells was proposed with the discovery of the seminal induction of neutrophil extracellular traps (NETs) (Alghamdi et al., 2004), which are DNA-based structures able to entrap pathogenic and non-pathogenic bodies (Brinkmann et al., 2004; Manfredi et al., 2018). Although the exact factors triggering this process are yet to be unveiled, neutrophil phagocytosis and NETosis are known to be tightly regulated (Branzk et al., 2014). For instance, phagocytosis, a faster process compared to NETosis, has been reported to sequestrate factors capable of inducing NETosis, such as neutrophil NE elastase (NE) and myeloperoxidase (MPO), into phagosomes (Branzk et al., 2014). In addition, although no consensus regarding the factors and signalling pathways involved in the induction of NET formation exists, particle size (Branzk et al., 2014) and reactive oxyge species (ROS) (Remijsen et al., 2011; Kaplan and Radic, 2012; Branzk et al., 2014; Papayannopoulos,

2018) have been pointed out as relevant. In any case, endometrial NETosis has been reported to be induced by both SP and sperm depending on the species; whereas they are triggered by sperm in humans (Piasecka et al., 2014) and pigs (Wei et al., 2020), SP seems to be crucial for NET formation and release in cattle (Alghamdi et al., 2009, 2010; Fichtner et al., 2020) and donkeys (Mateo-Otero et al., 2022). Sperm sequestration by NETs has been proposed to occur to selectively trap abnormal sperm, probably on the basis of sperm surface membrane features, rather than to reduce the access of sperm to oocytes (Tomlinson et al., 1992).

On the other hand, the second immune cells recruitment, involving **macrophages** and **dendritic cells**, has been recognised to promote uterine receptivity for embryo implantation. In particular, macrophages have been characterised to secrete enzymes and signalling molecules that: i) modify the luminal epithelial glycocalyx and stromal extracellular matrix to facilitate embryo attachment (Jasper et al., 2011) and trophoblast invasion during placentation (Robertson, 2005) and, ii) promote angiogenesis (Schäfer-Somi et al., 2013). Conversely, dendritic cells appear to have a long-term role, as they regulate the adaptive immune response by presenting antigens to T-cells. Thus, the main function of dendritic cells is to mediate immune tolerance to the paternal alloantigens that are later expressed by the implanting embryo to avoid immune-mediated abortions (Moldenhauer et al., 2009). In short, the inflammatory response triggered by seminal factors seems to be crucial to prepare the uterine environment for a successful implantation and to further support pregnancy (Schjenken and Robertson, 2020).

4.2.2. Influence of seminal factors on oviduct physiology and the establishment of the sperm reservoir

Similarly to the events occurring in the uterus, SP is also able to induce the release of cytokines after mating far from the semen deposition site (Bromfield et al., 2014; Álvarez-Rodríguez et al., 2020). In fact, in absence of SP, **oviductal cytokine expression** has been reported to be disrupted and embryo development success diminished in mice (Bromfield et al., 2014). The release of specific oviductal cytokines induced by SP is, therefore, likely to determine the success of the earliest stages of embryo development. Remarkably, oviduct physiology seems to be modulated not only by SP, but also by sperm. Interestingly, in pigs, the local immune response within the oviduct has been reported to rely upon which sex chromosome sperm carry and, thus, each type of sperm (i.e., X or Y) elicits a sex-specific signal transduction in oviductal cells (Almiñana et al., 2014).

In addition, SP has been found to facilitate the establishment of the **sperm reservoir** in the oviductal isthmus through two mechanisms. First, SP has been reported to have a direct effect on the oviductal sperm reservoir by inducing changes in the gene expression in both chickens and pigs (Atikuzzaman et al., 2017). Alternatively, SP proteins have also been described to facilitate sperm reservoir formation. An example of this can be found in cats, where the incubation of epididymal sperm with SP was seen to promote sperm attachment to oviduct explants (Henry et al., 2015). The molecular mechanisms regulating the establishment of the sperm reservoir have been described in depth in bovine and porcine species (Töpfer-Petersen et al., 2008; Talevi and Gualtieri, 2010). In bovine, BSP proteins from SP are able to attach to sperm head and, once in the oviduct, BSP-A1/2 can be recognised by receptors of the oviductal epithelium to form the sperm reservoir (Suarez, 2002; Gwathmey et al., 2003). A similar mechanism has been reported for pigs, in which spermadhesins from SP seem to be capable of binding the sperm surface and be recognised by oviductal

epithelial carbohydrates (Ekhlasi-Hundrieser et al., 2005). In addition, it is interesting to mention that pig sperm have also been reported to regulate the oviductal expression of genes that modulate the sperm function during the formation of the oviductal reservoir (Yeste et al., 2009, 2014).

Although many efforts have been made to specifically describe how seminal factors influence the uterine environment, the specific mechanisms underlying oviductal modulation are still unclear for many species. Yet, keeping in mind that SP is not likely to reach further than the uterus, only those factors from SP able to bind sperm can be hypothesised to influence oviductal physiology (Recuero et al., 2020). In any case, given that the oviductal events that semen seems to influence are of great relevance for the reproductive success, research on this particular topic might help to better explain early pregnancy failure in mammals.

4.2.3. Modulation of ovarian physiology by seminal factors

The SP seems to be able to control ovarian physiology in several mammalian species. First, SP has been reported to be indispensable to trigger ovulation in induced ovulatory species such as camels, alpacas, cats and rabbits (Adams and Ratto, 2013). Interestingly, the existence of a seminal factor regulating ovulation was demonstrated in experiments in which llama SP was administered to non-induced ovulatory species such as mice (Bogle et al., 2011) or cattle (Tanco et al., 2012) and ovulation was modified. In addition, in pigs, a non-induced ovulatory species, SP administration prior to ovulation was found to shorten the interval between LH peak and ovulation, as well as to increase P4 plasma concentration (Waberski et al., 1999; O'Leary et al., 2006). Besides ovulation, SP also appears to regulate CL function through the recruitment of ovarian macrophages in mice (Gangnuss et al., 2004) and pigs (O'Leary et al., 2006). The molecules accounting for CL formation have been presumed to access the uterine vein to reach the ovary and even the hypothalamic-pituitary axis

in the brain (Ratto et al., 2012). All these data suggest that SP, albeit not always essential for the reproductive success, facilitates ovulation and regulates CL function in mammals.

4.2.4. Seminal induction of embryotrophic/embryotoxic factors

Finally, semen has also been described to induce the release of factors capable of positively or negatively modulating embryo development (Robertson et al., 2011). Specifically, semen seems to be able to induce the uterine and oviductal secretion of these factors in species such as mice (Robertson et al., 1992; Bromfield et al., 2014) and pigs (O'Leary et al., 2004). In particular, a study in mice showed that the absence of SP upon mating resulted in the reduction of zygote cleavage and impaired blastocyst development (Bromfield et al., 2014). Similarly, in pigs, the administration of SP during artificial insemination (AI) was found to promote embryo development and to alter the uterine cytokine expression (O'Leary et al., 2004; Martinez et al., 2019).

Seminal factors are able to induce the release of **embryotrophic factors** by the female reproductive tract. Some of these factors are CSF1, CSF2, CSF3, IL-6, Leukemia Inhibitory Factor (LIF) and Vascular Endothelial Growth Factor (VEGF), and their effect is herein exemplified with CSF2. In response to semen exposure, the CSF2 released by the female reproductive tract targets pre-implantation embryos to promote blastocyst formation and enhance blastomere viability in mice (Robertson et al., 2001), regulate epigenetic reprogramming in cattle (Loureiro et al., 2009), and promote blastulation and increase ICM and TE cell numbers in humans (Sjöblom et al., 1999). On the contrary, under certain conditions, the uterus and oviduct can release **embryotoxic factors** that may compromise embryo development. The three embryotoxic factors known to be induced by SP are TNF, IFN-γ and TNF-related apoptosis inducing ligand (TRAIL). From those, TRAIL is the one whose effect has been better described. In particular, in mice (Riley

et al., 2004) and humans (Robertson et al., 2018), TRAIL binding to its receptor in preimplantation embryos has been reported to induce apoptosis.

Although the main *in vivo* interaction between semen and embryos has been described as indirect, via the modulation of gene expression in the maternal tract, a direct action of SP on embryo development should not be discarded. In fact, EVs could have a crucial role in this process, as a recent study carried out in mice observed that seminal EVs were able to improve *in vitro* embryo development (Ma et al., 2022). Yet, further studies should address the mechanisms underpinning the capacity of SP to control early embryo development.

5 Impact of male factors on fertility

As aforementioned, semen has been demonstrated to modify the female genital tract in order to create a suitable uterine environment enhancing reproductive success. For instance, beyond the activation of the female immune response, seminal factors seem to improve placental developmental, which directly influences foetal growth (Bromfield, 2014). Furthermore, seminal factors can also have an impact on embryo development, which may result in long-term consequences for offspring health. The molecular identity of these paternal factors, which are carried by both SP and sperm, is wide ranging, including proteins, lipids, nucleic acids, carbohydrates and other metabolites. Considering the aims of this Dissertation, the proteome, metabolome and sperm DNA impact on fertility are explained in more detail in the following sections.

5.1. Influence of sperm and seminal plasma molecules on male fertility potential

5.1.1. Seminal plasma and sperm proteome

The seek for molecular biomarkers able to explain sperm physiology and fertility potential began with the description of SP and sperm proteome. One reason for this interest is the transcriptionally and translationally inert nature of sperm (Baker and Aitken, 2009), which made the study of the seminal proteome the main research field for biomarkers for a long time. In this sense, the identification of proteins that might serve as fertility biomarkers became a relevant area of research, not only to understand how SP proteins are able to influence both sperm function and the female reproductive tract, but also from an applied perspective for fertility clinics and the animal breeding industry. In this regard, advanced proteomic techniques, including Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) and Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS), have represented a major scientific turning point in the field of reproductive biology, as they have allowed unravelling the complete proteome of both sperm and SP in many mammalian species (Druart et al., 2019). In fact, the widespread use and the improvement of the sensitivity of MS-based techniques over the last decade has led to both the identification of novel biomarkers able to predict sperm quality and fertility potential, and the validation of previously proposed markers (Agarwal et al., 2020b).

Earlier studies in humans identified candidate markers for specific clinical conditions, such as asthenozoospermia, azoospermia, oligoasthenozoospermia, globozoospermia, varicocele and even testicular cancer, in both sperm (Agarwal et al., 2020) and SP (Panner Selvam and Agarwal, 2018). Conversely, in livestock, research has been focused on how proteins present in sperm and SP are able to modulate sperm cryotolerance, physiology and fertility. Remarkably, some of these proteins have been

found to be common between species. An example of this are BSP and spermadhesins, which seem to be the most shared proteins between domestic animals, both having been repeatedly purported to modulate sperm maturation, metabolism and survival and to be associated to fertility in species such as pigs, sheep, cattle and horses (Druart et al., 2019). Notably, species-specific differences have also been reported; i) not all seminal proteins are shared across species (Druart et al., 2013); and ii) proteins that have been proposed to be relevant for the reproductive strategy in one species are often not in others. Focusing on the latter, this can be exemplified with proteins modulating ovulation, particularly the ovulation inducing factor β-nerve growth factor (β-NGF). This protein has been detected in the SP of both induced and spontaneous ovulators, suggesting that it may or may not play a crucial role in ovulation induction depending on the species (Suarez and Wolfner, 2017). For this reason, although high throughput proteomic studies have driven the identification of potential biomarkers, further research is needed to elucidate the exact mechanisms by which proteins from both SP and sperm might be influencing sperm performance, and the potential particularities in each species. This is further approached in this Dissertation, particularly regarding the role of Aldose Reductase B 1 (AKR1B1) in reproductive physiology.

Role of AKR1B1 in mammalian reproduction

Aldose reductases are NADPH-dependent enzymes that belong to the aldo-keto reductase (AKR) superfamily (Bohren et al., 1989; Hyndman et al., 2003). The AKRs are divided into 16 families of monomeric (Jez et al., 1997) and multimeric forms (Kavanagh et al., 2002; Kozma et al., 2002; Barski et al., 2008). In particular, AKR1B1 belongs to the AKR family 1, the largest one, which also includes proteins such as aldehyde reductases, hydroxysteroid dehydrogenases and steroid 5β-reductases (Hyndman et al., 2003). It is the

most studied aldose reductase, probably because of its relevant function in cell homeostasis, specifically: i) as a part of the polyol pathway involved in fructose production, catalysing the conversion of glucose into sorbitol (Avancini and Rossing, 2015); and ii) as a detoxification enzyme through the reduction of carbonyl-containing metabolic compounds (Srivastava et al., 2005).

This protein has been described to be relevant for both male and female reproductive physiology in several mammalian species, including humans (Bresson et al., 2011), cattle (Frenette et al., 2004; Girouard et al., 2009), rats (Kobayashi et al., 2002), mice (Jagoe et al., 2013), sheep (Yang et al., 2019) and pigs (Steinhauser et al., 2016). Regarding male reproductive physiology, AKR1B1 has been reported to contribute to epididymal maturation in cattle (Frenette et al., 2003) and mice (Jagoe et al., 2013). On the other hand, porcine aldose reductase has been proposed to modulate sperm fertilising ability via the regulation of sperm motility during capacitation (Katoh et al., 2014). In addition, a detoxifying function of AKR1B1 has been shown in rodents, which is suspected to contribute to sperm survival (Kobayashi et al., 2002; Jagoe et al., 2013). Besides, a study conducted by Pérez-Patiño et al. (2018) performed an in-depth proteomic analysis of pig SP to find potential *in vivo* fertility biomarkers and pointed out AKR1B1 as one of the most relevant (Pérez-Patiño et al., 2018). Specifically, the levels of AKR1B1 in SP were found to be higher in boars with high farrowing rates compared to those with low ones. Given its great potential, the role played by SP-AKR1B1 in male reproductive physiology was further investigated in this Thesis Dissertation.

Regarding female reproductive physiology, AKR1B1 has been reported to act as a prostaglandin F synthase in the endometrium of humans (Bresson et al., 2011), cattle (Madore et al., 2003) and pigs (Seo et al., 2014) during early pregnancy. In particular, AKR1B1 has been found to directly synthesise PGF2α (Bresson et al., 2011) and indirectly influence

PGE2 production (Bresson et al., 2012). As briefly mentioned above, PGF2α is a widely known luteolytic factor (Jensen et al., 1987). In contrast, PGE2 has been described to maintain luteal function for early embryo development and to promote implantation (Niringiyumukiza et al., 2018).

Finally, AKR1B1 has also been identified in embryos. In effect, AKR1B1 was detected in conceptus-derived exosomes of pregnant cows at Days 15 and 17 of gestation (Nakamura et al., 2016). In contrast, *AKR1B1* transcripts were found to be enriched in blastocysts that did not result in pregnancy or were resorbed (El-Sayed et al., 2006). Thus, the involvement of this protein on fertility in terms of uterine environment modulation and embryo development still needs to be clarified.

5.1.2. Seminal plasma and sperm metabolome

The metabolome can be defined as the total set of small molecular weight compounds, known as metabolites, that participate in catabolic and anabolic pathways. In fact, metabolites are the end products of cellular metabolic processes and biological systems (Goodacre et al., 2004). Metabolites are thus considered to reflect the downstream events of gene expression, amplifying the potential changes in response to various conditions compared to transcriptome and proteome (Deepinder et al., 2007). In the last decades, the progress in analytical technologies, including MS and Nuclear Magnetic Resonance (NMR) spectroscopy, have allowed the identification of novel biomarkers in a wide range of biofluids, including semen.

The metabolomic profile of both SP and sperm has been analysed in several mammalian species in recent years. Most studies have been focused on SP, probably due to the challenge the reduced cytoplasm of sperm represents for metabolite extraction and because, in most species, getting sufficient SP volume is easier than harvesting enough sperm cells

for their analysis. Yet, studies conducted in humans (Zhao et al., 2018; Engel et al., 2019) and cattle (Menezes et al., 2019) were able to characterise the sperm metabolome and suggested a relationship with *in vivo* fertility outcomes. On the other hand, the relationship between SP metabolites and sperm physiology was explored in humans (Wang et al., 2019), pigs (Mateo-Otero et al., 2021) and sheep (Jia et al., 2021). In addition, similarly to proteomic studies conducted in humans, the metabolome of both SP and sperm was characterised for several pathological conditions such as asthenozoospermia (Li et al., 2020), oligoasthenoteratozoospermia (Mumcu et al., 2020) and azoospermia (Bonechi et al., 2015; Gilany et al., 2017). In livestock, most studies have been carried out in bulls, where differences in the metabolomic profile were reported between individuals with different fertility (Kumar et al., 2015; Velho et al., 2018; Talluri et al., 2022). While the metabolite content of SP seems to be conserved across species (Gupta et al., 2011; Bonechi et al., 2015; Kumar et al., 2015; Mateo-Otero et al., 2020), the relationship between certain metabolites and sperm physiological traits and fertility potential seems to be species-specific.

The vast majority of metabolomic characterisations of both SP and sperm have followed an untargeted approach in order to provide a complete metabolite profile and explore the relevance of specific metabolites as potential biomarkers. That being said, more research is still needed not only to validate the detected relationships, but also to investigate in more detail specific pathways that may impact on reproductive events. For instance, targeted metabolomic approaches could be useful for this purpose, as they measure defined groups of annotated metabolites to characterise specific pathways (Roberts et al., 2012). In the light of the above, this was further investigated in the present Doctoral Thesis. Particularly, and to explore the relationship between metabolites and fertility outcomes, two approaches were followed: a) an untargeted characterisation of the SP metabolome; and b) a targeted characterisation of sperm metabolites.

5.1.3. Seminal extracellular vesicles

Extracellular vesicles are nano-sized lipid organelles shed by cells that act as a vehicle for intercellular communication, both locally and remotely. The EVs are capable of packaging intracellular molecular components involved in signalling, including proteins, metabolites, lipids, cytokines and RNA, among others. These EV-packed molecules are able to modify the targeted cells function in both physiological and pathological conditions (Machtinger et al., 2016). Based on their biogenesis and size, EVs can be classified as: i) exosomes (50-150 nm), intraluminal vesicles resulting from the endosomal pathway; ii) microvesicles (100-1000 nm), formed by plasma membrane budding; and iii) apoptotic bodies (500-4000 nm), blebs originated from cells undergoing apoptosis. These three types of EVs also seem to differ in their cargo. While exosomes and microvesicles carry molecules that participate in cell-cell communication, cell maintenance and tumour progression, apoptotic bodies contain nuclear fragments and cell debris. Furthermore, and unlike apoptotic bodies, exosomes and microvesicles are characterised by protein markers including tetraspanins and adhesion integrins in their plasma membrane (Van Niel et al., 2018). The identification of EVs in reproductive biofluids such as SP, and oviductal and uterine fluids and even *in vitro* embryo culture (IVC) media has completely changed the paradigm of how male-female and female-embryo interactions work (Machtinger et al., 2016).

Focusing on the male, the SP is one of the biological fluids with the highest content of EVs, compared to the cerebrospinal fluid or blood plasma (Skalnikova et al., 2019). Although the relative contribution of microvesicles and exosomes to the EVs present in SP still needs to be elucidated, it has been calculated that 10% of the total protein content of SP is encapsulated in EVs (Panner Selvam and Agarwal, 2018). Traditionally, seminal EVs have been understood as a mixture of EVs released by secretory acinar cells of the prostate and by the epididymal epithelium, leading to naming these EVs as “prostasomes” and “epididymosomes”, respectively (Sullivan and Saez,

2013; James et al., 2020). Yet, other regions of the male reproductive tract have also been reported to release EVs, including Sertoli cells of the testis, epithelial cells of seminal vesicles and ductus deferens (Roca et al., 2022). Seminal EVs should, therefore, be understood as a heterogeneous population of EVs originated all along the male reproductive tract.

As mentioned in Section 1.1.3 of the Introduction, epididymosomes are able to modulate sperm maturation during their transit throughout epididymis (Suryawanshi et al., 2012; Caballero et al., 2013; James et al., 2020). Upon ejaculation, seminal EVs interact with both sperm and the female reproductive tract, as reported in several mammalian species. First, the interaction of seminal EVs with sperm seems to regulate different physiological processes, such as maturation, motility, capacitation and the acrosome reaction. The exact role of seminal EVs is, nevertheless, still under debate, as published data appear to be inconsistent. On the one hand, studies conducted in pigs reported that seminal EVs are able to inhibit cholesterol efflux and keep sperm plasmalemma intact, thereby delaying or even inhibiting sperm capacitation in pigs (Piehl et al., 2013; Du et al., 2016) and humans (Pons-Rejraji et al., 2011). This was further confirmed by Bechoua et al. (2011), who reported that incubation of human sperm with prostasomes decreases tyrosine phosphorylation levels, one of the most common capacitation markers (Bechoua et al., 2011). In contrast, other investigations found that seminal EVs are able to potentiate sperm capacitation in pigs (Siciliano et al., 2008) and humans (Murdica et al., 2019). Moreover, seminal EVs also seem to influence the female reproductive tract in terms of immune regulation, in several species including pigs (Bai et al., 2018), humans (Kelly et al., 2008) and cattle (Lazarevic et al., 1995). Although all these findings clearly demonstrate the essential role of seminal EVs in reproductive physiology, further research to unravel the exact contribution of free and EV-encapsulated molecules in such processes is much warranted.

5.2. Influence of sperm chromatin structure on fertility outcomes

Sperm DNA has been reported to influence reproductive outcomes beyond the effect of its genome on progeny. For instance, chromatin structure, which comprises both nucleoproteins and chromatin integrity, has been described as a key factor in the regulation of mammalian embryo development. Altered histone content, histone/protamine ratio, protamine 1/protamine 2 ratio and epigenetic signatures result in abnormal chromatin packing, which detrimentally affects embryo development in humans (Castillo et al., 2015; Barrachina et al., 2018; Fournier et al., 2018). In fact, aberrant nucleoprotein content and/or distribution has also been proposed to increase DNA susceptibility to damage and abnormal epigenetic marking (Barrachina et al., 2018).

Sperm DNA fragmentation is a genotoxic insult that mainly occurs during spermatogenesis and sperm transport through the male reproductive tract. Specifically, sperm DNA damage acquired throughout spermatogenesis may be caused by apoptosis in the seminiferous tubules or defects over chromatin remodelling during spermiogenesis. Regarding sperm transport, the most common mechanisms triggering DNA damage are oxygen radicals produced during sperm migration along the epididymis, the activation of sperm caspases and endonucleases, environmental toxicants, chemotherapy and radiotherapy (Sakkas and Alvarez, 2010).

Two types of DNA breaks have been characterised in sperm: single- and double-strand DNA breaks. **Single-strand breaks** are mainly caused by free radicals, which can oxidise DNA bases into 8-OH-guanine and 8-OH-2'-deoxyguanosine destabilising the DNA structure and causing DNA breaks (De Iuliis et al., 2009; Santiso et al., 2010). The reactive oxygen species (ROS) that cause single-strand breaks can have both an exogenous origin, such as environmental toxins and alcohol, and/or endogenous origin, such as

increased mitochondrial activity or varicocele (Aitken and De Iuliis, 2010; Sakkas and Alvarez, 2010; Agarwal et al., 2014). Single-strand breaks are usually widespread in the whole sperm genome, as they occur in both protamines-compacted toroidal regions and histones-compacted matrix attachment region (MAR) regions (Ribas-Maynou et al., 2012). On the other hand, **double-strand breaks** occur during spermatogenesis, the histone-protamine exchange and the activation of apoptosis (Agarwal et al., 2020a). This DNA damage tends to be more localised, specifically in MAR regions (Ribas-Maynou et al., 2012).

Sperm DNA damage can be assessed through different techniques. The traditional assays include Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion (SCD) test, which cannot decipher the type of DNA break or the region affected. There is, however, another methodology, the Comet assay, that has higher sensitivity and can discriminate between single-strand breaks and total DNA damage (Ribas-Maynou and Benet, 2019).

The presence of unrepaired DNA breaks has been found to affect *in vitro* and *in vivo* fertility outcomes in several mammalian species. For instance, the effect of DNA breaks on fertility has been named as late paternal effect (Tesarik, 2005), as embryos with normal karyotype but bearing these DNA breaks fail to develop further than morula or blastocyst stages. This embryo developmental arrest has been found to occur in two ways in humans: a) fertilised oocytes are unable to reach late developmental stages after embryo genome activation; and b) embryos fail to further develop from morula or blastocyst stage and/or implant (Sakkas and Alvarez, 2010; Ribas-Maynou et al., 2021). Yet, considering that this needs to be validated in other mammals, the present Dissertation also addressed how sperm DNA damage influences oocyte fertilisation and embryo development.

5.3. Male factors impact on offspring health

It is known that the two progenitors contribute to the offspring phenotype, mainly through the information encoded in the genome. In the case of the male, where sperm are accompanied by SP upon mating, it is possible that factors other than the DNA play a role and determine offspring health. Indeed, besides its relevance in the modulation of the maternal environment, seminal factors have been described to influence foetal development long after oocyte fertilisation and pre-implantation embryo stages.

The impact of seminal factors on offspring health was first tested in hamsters, where the excision of paternal accessory sex glands resulted in reduced postnatal growth and increased anxiety in adult pups (Wong et al., 2007). Later, in mice, the offspring from females that had not been exposed to seminal vesicle secretions during mating showed placental hypertrophy and, after birth, symptoms of metabolic syndrome including increased adiposity and hypertension, particularly in the male progeny (Bromfield et al., 2014). These authors hypothesised that the effect of these secretions on phenotype was likely to be explained by epigenetic mechanisms, which would be supported by studies linking the embryo epigenome to metabolic disorders (Seki et al., 2012). In addition, regarding the observed higher incidence of metabolic syndrome symptoms in the male progeny, several studies previously reported different epigenetic modifications between sexes at preimplantation stages, particularly showing that male embryos are more sensitive to environmental stress during IVC, later affecting embryo development and possibly offspring health (Sjöblom et al., 2005; Bermejo-Alvarez et al., 2011). All these findings suggest that male accessory glands secretions may have the potential to affect offspring long after conception and embryonic development in a sex-specific manner.

Next, a more recent work sought to investigate the exact contribution of both SP and sperm to offspring health in mice (Watkins et

al., 2017). In that study, it was reported that mice derived from semen of males fed with a low protein diet were heavier than those derived from semen from males fed with a normal protein diet. Interestingly, when sperm from animals fed with normal protein content diets were mixed with the SP from animals fed with low protein diets, the offspring displayed alterations in growth and hepatic gene expression (Watkins et al., 2018). From these results, the authors concluded that while sperm may transfer genetic/epigenetic information, the effect of SP might be concentrated in the modulation of maternal environment (Morgan and Watkins, 2019). In spite of this, it cannot be excluded that the impaired offspring health could be caused by newly introduced epigenetic modifications to sperm DNA.

Finally, in addition to seminal factors, paternal well-being at the time of conception has also been reported to influence long-term offspring health. For instance, the offspring of male mice fed with a low-protein diet was found to have increased expression levels of cholesterol and lipid synthesis-related genes in their livers (Carone et al., 2010). Similarly, feeding rodents with high fat or low folate diets or even paternal fasting was found to compromise offspring metabolic health (Anderson et al., 2006; Fullston et al., 2013; Lambrot et al., 2013; Cropley et al., 2016). All these works also pointed out the epigenome as the primary vehicle for the transmission of these alterations. More importantly, all these studies introduced a new variable in the factors shaping the reproductive success in the long term: the environmental factors. Either way, epigenetic signatures seem to be capable of reprogramming the genome so that novel features can be inherited through the mammalian germline.

Objectives

The repercussion of paternal factors on fertility seems to comprise much more than the information encoded in the sperm DNA. For instance, both SP and sperm appear to influence the reproductive success, directly and indirectly. In view of the above considerations, **the general aim of this Dissertation was to increase the current knowledge about the contribution of seminal factors to *in vivo* and *in vitro* fertility outcomes in mammals**. For this purpose, two Chapters were devised, with the following specific objectives and sub-objectives:

Chapter 1

To address whether SP can modulate *in vivo* fertility, and the potential molecular mechanisms underlying this regulation:

1. To evaluate whether SP can influence preimplantation embryo development *in vivo*. **- Paper I -**
2. To explore the mechanisms by which SP-proteins modulate *in vivo* fertility, using AKR1B1 as a candidate marker. **- Paper II -**
3. To examine whether SP metabolites may affect *in vivo* fertility outcomes. **- Paper III** –

Chapter 2

To investigate sperm molecular mechanisms able to modulate *in vitro* fertility, in terms of oocyte fertilisation and pre-implantation embryo development:

1. To determine the mechanisms by which sperm proteins modulate sperm function, oocyte fertilisation and embryo development, using AKR1B1 as a candidate marker. **- Paper IV -**
2. To describe how sperm metabolism may impact *in vitro* fertility outcomes. **- Paper V -**
3. To assess whether the integrity of sperm DNA has any role on the reproductive success evaluated *in vitro*. **- Paper VI -**

Chapter 1

Seminal plasma modulates the female environment and sperm physiology

Paper I

Effect of exposure to seminal plasma through natural mating in cattle on conceptus length and gene expression.

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Effect of Exposure to Seminal Plasma Through Natural Mating in Cattle on Conceptus Length and Gene Expression

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A growing body of evidence suggests that paternal factors have an impact on offspring development. These studies have been mainly carried out in mice, where seminal plasma (SP) has been shown to regulate endometrial gene expression and impact embryo development and subsequent offspring health. In cattle, infusion of SP into the uterus also induces changes in endometrial gene expression, however, evidence for an effect of SP on early embryo development is lacking. In addition, during natural mating, the bull ejaculates in the vagina; hence, it is not clear whether any SP reaches the uterus in this species. Thus, the aim of the present study was to determine whether SP exposure leads to improved early embryo survival and developmental rates in cattle. To this end, Day 7 *in vitro* produced blastocysts were transferred to heifers (12–15 per heifer) previously mated to vasectomized bulls (n = 13 heifers) or left unmated (n = 12 heifers; control). At Day 14, heifers were slaughtered, and conceptuses were recovered to assess size, morphology and expression of candidate genes involved in different developmental pathways. Additionally, CL volume at Day 7, and weight and volume of CL at Day 14 were recorded. No effect of SP on CL volume and weight not on conceptus recovery rate was observed. However, filamentous conceptuses recovered from SP-exposed heifers were longer in comparison to the control group and differed in expression of *CALM1*, *CITED1*, *DLD*, *HNRNPDL*, *PTGS2*, and *TGFB3*. In conclusion, data indicate that female exposure to SP during natural mating can affect conceptus development in cattle. This is probably achieved through modulation of the female reproductive environment at the time of mating.

Keywords: seminal plasma, embryo development, corpus luteum, cattle, gene expression

INTRODUCTION

Despite the molecular complexity underlying the critical processes that take place in the peri-conception and early preimplantation period, the success of *in vitro* reproductive techniques suggests that the requirements of the embryo can be met by a relative simple set of media, and that embryonic development, at least to the blastocyst stage, occurs independently of interaction

with the female reproductive tract. While blastocyst stage embryos can induce changes in the endometrium (Sponchiado et al., 2017; Passaro et al., 2018, 2019) as well as the uterine lumen metabolite composition (Sponchiado et al., 2019), mainly through embryo-derived interferon-tau (Passaro et al., 2019), whether this interaction plays an important role in embryo survival is questionable given the fact that embryos can be transferred to a virgin uterus as late as Day 16 and still establish a pregnancy (Betteridge et al., 1980). Nonetheless, it is becoming apparent that offspring health can be affected by the environmental conditions experienced during gamete maturation, embryo development and fetal growth (Hanson and Gluckman, 2014). While the link between maternal environment and embryo and offspring wellbeing has been investigated in detail, the role of paternal factors in directing offspring development has been somewhat overlooked (Morgan and Watkins, 2019). However, there is growing evidence that paternal nutrition and body mass composition have direct impact on DNA integrity, sperm quality and epigenetic status (Fleming et al., 2018), which has an effect on the metabolic function of the offspring in mice (Bromfield et al., 2014; Watkins and Sinclair, 2014; Watkins et al., 2017, 2018). In addition, seminal plasma (SP) has been shown to modulate gene expression and the immune response of the female reproductive tract in some species such as mice, human and cattle (Fazeli et al., 2004; Sharkey et al., 2007; Chen et al., 2014; Ibrahim et al., 2019).

Seminal plasma, a fluid resulting primarily from the secretions of the male accessory glands, transports, nourishes and protects sperm at the time of ejaculation (Bromfield, 2016). At this time, sperm are coated by SP proteins that are believed to prevent capacitation until they are close to the oocyte, and that modify sperm metabolism and motility (Vicens et al., 2014).

Female exposure to SP has been shown to improve embryo development and survival in mice (Bromfield et al., 2014), humans (Crawford et al., 2015), pigs (O'Leary et al., 2004) and golden hamsters (O et al., 1988). In addition, in llamas and rabbits, species with induced ovulation, SP has been reported to stimulate ovulation through nerve growth factor (Silva et al., 2011; Ratto et al., 2012, 2019; Adams and Ratto, 2013). It is thought that the beneficial effect of SP on the embryo is due in part to the immunoregulatory role. Indeed, leukocyte infiltration in response to SP in mice (Hunt and Robertson, 1996; Tremellen et al., 1998) and pigs (O'Leary et al., 2004) has been observed. Traditionally, this migration of immune cells was thought to solely serve the purpose of clearing microorganisms and excess sperm (O'Leary et al., 2004). However, it has been demonstrated that seminal Transforming Growth Factor Beta (TGFβ) stimulates Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) secretion by uterine epithelial cells *in vivo* and *in vitro* in mice (Robertson et al., 1997; Tremellen et al., 1998; Moldenhauer et al., 2010). This factor promotes a pro-inflammatory cytokine and chemokine cascade, which recruits immune cells into the endometrial lumen and induces differentiation of tolerogenic dendritic cells and regulatory T cells (Treg cells) (Robertson et al., 2013). This differentiation depends on the micro-environmental cytokine signals which control the transition of naïve Th0 cells into Treg cells. As a result, immune tolerance to paternal antigens is probably established, which improves the ability of the semi-allogenic embryo to implant and develop normally (Robertson et al., 2009, 2018; Guerin et al., 2011). In addition to facilitating embryo implantation, in the mouse, exposure to semen induces the oviductal and uterine synthesis of embryotrophic cytokines such as GM-CSF, Interleukin 6 (IL6) and Leukaemia Inhibitory Factor (LIF) (Robertson, 2007). In this species, these factors have been shown to improve fetal growth and placentation (Sjöblom et al., 2005). Finally, regulation of ovarian function by SP has also been reported. In mice, the macrophage population in the corpus luteum (CL) has been observed to increase one day after mating in response to uterine exposure to SP (Gangnuss et al., 2004). Similarly, an increase in CL size and progesterone (P4) concentration in peripheral blood after SP exposure in pigs has been reported (O'Leary et al., 2006). Elevated P4 during the preimplantation stage has been shown to positively influence embryo growth in mice (Aisemberg et al., 2013), ruminants (Kleemann et al., 1994; Inskeep, 2004; Clemente et al., 2009; O'Hara et al., 2014a), alpacas (Bravo and Diaz, 2010), and pigs (Ashworth, 1991; Jindal et al., 1997). This is, at least in part, due to P4-stimulated endometrial secretions, collectively termed histotroph, which support conceptus development, implantation, and placentation (Simintiras et al., 2019). Thus, the SP-induced increase in P4 likely benefits embryo development and subsequent survival.

Despite the extensive body of evidence in the mouse, in cattle, the effect of SP on embryo development and survival is not clear. Both sperm and SP have been shown to induce expression of pro-inflammatory-related genes in the endometrium after infusion of SP into the bovine uterus (Elweza et al., 2018; Ibrahim et al., 2019; Ortiz et al., 2019). However, using a similar infusion technique, no improvement in the fertility of dairy heifers (Odhiambo et al., 2009) or cows (Ortiz et al., 2019) was observed. These apparent inter-species differences in the role of SP in fertility may be due, in part, to the known variation in composition of this fluid (Rodger, 1976; Druart et al., 2013), owing to differences in accessory gland size, type and level of fluid contribution to the ejaculate (Bedford, 2015). Indeed, caution is needed when interpreting some of the aforementioned bovine studies as some used SP collected by electroejaculation (Ibrahim et al., 2019), others by artificial vagina (Odhiambo et al., 2009) and others do not mention the collection method (Elweza et al., 2018; Ortiz et al., 2019). Collection method significantly influences the composition of SP (Rego et al., 2015) and consequently has a tremendous impact on endometrial response *in vitro* (Fernandez-Fuertes et al., 2019). As a result of these issues, interpretation of the available data in cattle is challenging.

In addition, the site of ejaculate deposition (i.e., intravaginal or intrauterine) likely determines differences in the response of female reproductive tissues to SP. Due to the characteristics of mating in rodents (Dean et al., 2011) and pigs (Hunter, 1981), SP comes into direct contact with the uterus. In contrast, during natural mating, the bull deposits the ejaculate in the anterior vagina of the cow (Hawk, 1983). Thus, it is not clear if any SP reaches the uterus in this species. However, at ejaculation, SP proteins bind to the sperm membrane (Pini et al., 2016), and

can therefore be carried by these cells into more distal regions of the female reproductive tract. In fact, some SP proteins that bind to sperm, such as Binder of Sperm Protein (Suarez and Pacey, 2006) and osteopontin (Souza et al., 2008), have previously been described to influence embryo development *in vitro* in pigs (Hao et al., 2008) and cattle (Gonçalves et al., 2008; Monaco et al., 2009; Rodríguez-Villamil et al., 2016). However, although bovine sperm can bind to both endometrial and oviductal cells *in vitro* and stimulate mRNA expression of different cytokines (Yousef et al., 2016; Elweza et al., 2018; Ezz et al., 2019), incubation of endometrial explants with cauda epididymis sperm (which are mature but have had no contact with SP) or Percoll-washed ejaculated sperm did not induce differences in mRNA expression of some of those cytokines (Fernandez-Fuertes et al., 2019). Thus, it is not clear if SP-derived proteins, rather than intrinsic sperm proteins, are responsible for eliciting changes in the endometrium of cattle.

It is also possible that, in species that deposit semen in the vagina, SP elicits a local response that propagates to more distal regions of the female reproductive tract. In humans, unprotected vaginal coitus leads to enhanced cytokine and chemokine expression in the cervix (Sharkey et al., 2012). These factors could travel through local circulation to elicit changes in other reproductive organs, as seems to be the case in pigs, rodents, and cattle. In the bovine, SP infusion into the vagina, but not the uterus, induces an increase in endometrial epidermal growth factor concentrations (Badrakh et al., 2020). Whereas mating (in the mouse) or infusion of SP (in the sow) have been shown to have an effect on ovarian function (Gangnuss et al., 2004; O'Leary et al., 2006).

Taken collectively, the literature suggests that although SP is not essential for pregnancy success, it can improve embryo development and survival through modulation of the maternal environment. However, to the best of our knowledge, there is currently no evidence for a role of SP in early embryo development in cattle *in vivo*. Thus, the aim of the present study was to assess the effect of SP exposure in cattle through natural mating on pre-implantation embryo survival and conceptus development. To this end, a model in which heifers were mated to vasectomized bulls (which only ejaculate SP) or left unmated (control) was used. *In vitro* produced embryos were transferred to the heifers at Day 7 post-mating and were recovered after slaughter at Day 14 to assess conceptus size, morphology and gene expression. In addition, CL volume at Day 7, as well as weight and volume at Day 14 were analyzed.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College Dublin, Ireland, and the Universitat de Girona, Spain, and licensed by the Health Products Regulatory Authority (HPRA), Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes). Throughout the course of the experiment, all animals were housed at Teagasc Grange, Animal and Grassland Research Centre, Dunsany, Ireland.

Experimental Design

The estrous cycles of crossbred beef heifers (mainly Angus and Holstein-Friesian cross; n = 27) were synchronized using an 8-day intravaginal device (PRID® Delta, 1.55 g progesterone; Ceva Santé Animale; Libourne, France), together with a 2 mL intramuscular injection of a synthetic gonadotrophin releasing hormone (Ovarelin®, equivalent to 100 μg Gonadorelin; Ceva Santé Animale) administered on the day of PRID insertion. One day prior to PRID removal, all heifers received a 5 mL intramuscular injection of PGF2α (Enzaprost®, equivalent to 25 mg of Dinoprost; Ceva Santé Animale) to induce luteolysis. Only heifers observed in standing estrus (Day -1; n = 25) were blocked by weight and randomly allocated to one of two treatments: (1) mated with a vasectomized bull (n = 13), or (2) left unmated (control; n = 12). Each mated heifer was hand-mated once to one of three vasectomized Holstein Friesian bulls within 7 h of the start of standing estrus. Bulls were allowed to mate no more than twice per day and the experiment was carried out over three consecutive days. Seven days after mating, *in vitro* produced blastocysts were transferred to each heifer (n = 12–15 per heifer). All heifers were slaughtered in a commercial abattoir 7 days after embryo transfer to recover Day 14 conceptuses. In addition, CL volume at Day 7, and CL weight and volume at Day 14 were recorded. The experimental design is summarized in **Figure 1**.

In vitro Embryo Production

Ovaries from cows and heifers were collected at a commercial abattoir and surface visible follicles (>2 mm) were aspirated to recover cumulus-oocyte complexes (COCs). Good quality COCs were matured in TCM-199 (Sigma Aldrich, Saint Louis, MO, United States) supplemented with 10% (v/v) FCS (Sigma Aldrich) and 10 ng/mL Epidermal Growth Factor (Merck; Darmstadt, Germany) (n = 50 COCs per well) for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured COCs were fertilized using sperm from a bull of proven *in vitro* fertility at a concentration of 1×10^6 sperm/mL. Frozen-thawed semen from the same bull was used throughout. Motile sperm were selected by centrifugation through a 95–45% discontinuous Percoll gradient (Merck) for 10 min at 700 g, followed by a second centrifugation in HEPES-buffered Tyrode medium (Boston BioProducts; MA, United States) at 100 g for 5 min. Gametes were co-incubated at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. Approximately 20 h post-insemination, cumulus cells were removed, and presumptive zygotes were cultured in 25 μL droplets of synthetic oviduct fluid supplemented with 5% FCS (n = 25 per well) under mineral oil (Sigma Aldrich) until Day 7 (Day 0 = day of fertilization). Culture dishes were kept at 39°C under an atmosphere of 5% CO_2 and 5% O_2 in air with maximum humidity until Day 7. Blastocysts classified as excellent or good (following guidelines of the International Embryo Technology Society, 2009) were pooled, and then randomly loaded into straws (n = 12–15 embryos/straw) for embryo transfer.

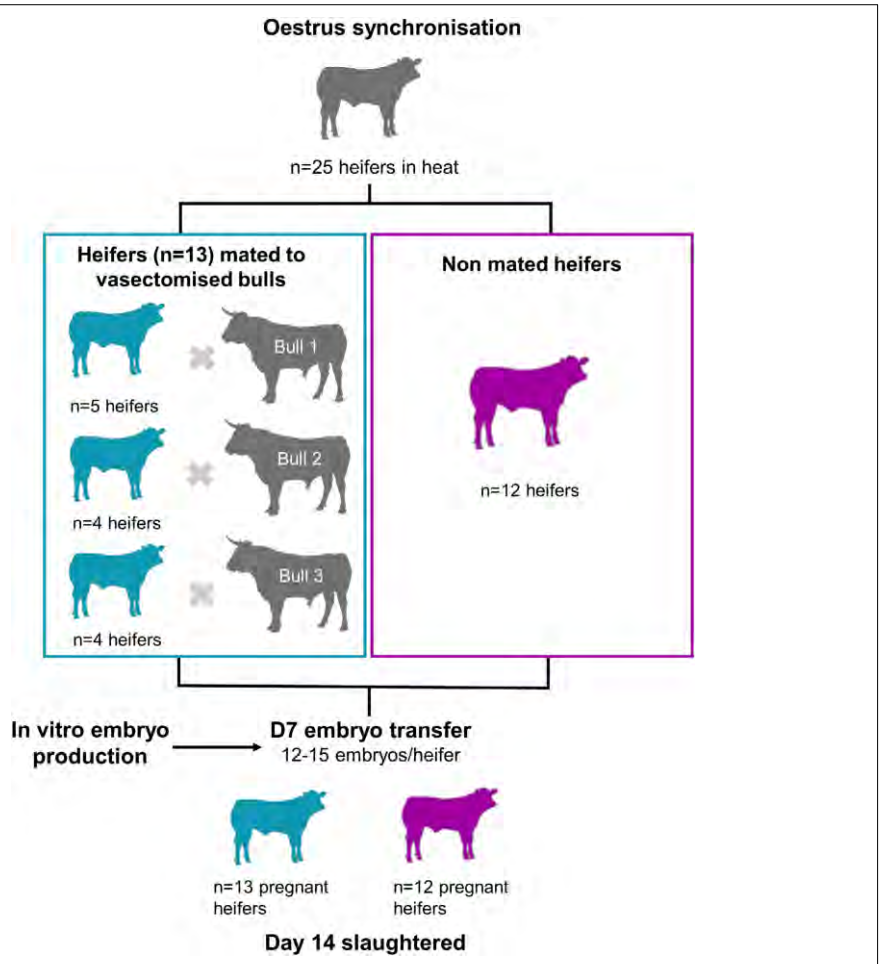

FIGURE 1 | Experimental design. Heifers observed in standing estrus (n = 25) were blocked by weight and randomly allocated to one of two treatments: mated with vasectomised bulls (blue) or left un-mated (purple). Seven days later, 12–15 *in vitro* produced blastocysts were transferred to all heifers. Heifers were slaughtered at Day 14, and the conceptuses were recovered from the uterine horns.

Embryo Transfer and Recovery

On Day 7 (Day 0 = day of *in vitro* fertilization for the embryos, and day after mating for the heifers) the Day 7 *in vitro*-produced blastocysts were transferred (n = 12–15 blastocysts/heifer) to the horn ipsilateral to the ovary bearing the CL. All heifers were slaughtered on Day 14 (7 days after embryo transfer). Reproductive tracts were recovered, gently dissected, and flushed with PBS containing 5% FCS within 30 min of slaughter. The number and dimensions (length and width) of recovered Day 14 embryos were recorded. Conceptuses were classified based on morphology as ovoid (1–4 mm), tubular (5–19 mm), or filamentous (>20 mm), based on previous studies (Ribeiro et al., 2016). Due to the large range in conceptus length in the filamentous group, the filamentous group was subdivided in short filamentous (20–25 mm) and long filamentous (>25 mm) for gene expression analysis.

Calculation of CL Weight and Volume

Corpus luteum volume was calculated as described previously (Scully et al., 2014; Parr et al., 2017). Briefly, for Day 7 CL, the formula for the volume of a sphere was used ($V = 4/3\pi r^3$). The radius was calculated as the average of the two cross-sectional

ultrasound measurements (the CL diameter) divided by two. For those CL with a cavity, the volume of the cavity was calculated using the same formula and subtracted from the total CL volume.

Because measurements in three axes (a, b, c) could be taken from post-mortem Day 14 CL, the formula for the volume of an ellipsoid ($V = 4/3\pi abc$) was used (Grygar et al., 1997). As above, for CL with a cavity, the volume of the cavity was subtracted from the total CL volume. Moreover, the weight of luteal tissue of these CL was also recorded.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR (RT-qPCR) was used to investigate changes in relative abundance of candidate transcripts in all Day 14 conceptuses due to treatment. A panel of five genes was used to determine conceptus gastrulation stage based on Degrelle et al. (2011): Calmodulin 1 (*CALM1*), Cbp/P300 interacting transactivator with Glu/Asp rich carboxy-terminal (*CITED1*), Dihydrolipoamide Dehydrogenase (*DLD*), Heterogeneous Nuclear Ribonucleoprotein D Like (*HNRNPDL*), and Transforming Growth Factor Beta 3 (*TGFB3*). However, under the conditions of the current study, the gene expression patterns that the authors described to classify gastrulation stage were not observed. Because the aforementioned genes are involved in different functional pathways within the conceptus, additional genes that participate in such pathways, and that have been found to be differentially expressed along development (Mamo et al., 2011; Barnwell et al., 2016) were interrogated: Caspase 3 (*CASP3*), Furin (*FURIN*), Glutathione S-Transferase Mu 1 (*GSTM1*), *IL6*, MHC Class I JSP 1 (*JSP1*), and Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*).

Total RNA was extracted from entire conceptuses using Trizol reagent (Invitrogen; Carlsbad, CA, United States) and trimethylene chlorobromide (Sigma Aldrich). On-column DNase digestion and RNA clean-up was performed using the RNeasy Mini Kit (Qiagen; Hilden, Germany) following the manufacturer's instructions. The quantity and purity of RNA was determined using the Epoch Microplate Spectrophotometer (BioTek; Winooski, VT, United States). For each sample, cDNA was prepared from 14.7 ng of total RNA (based on the sample with lowest RNA concentration) using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific; Waltham, MA, United States) according to the manufacturer's instructions. For the PCR negative control, a retrotranscription mastermix without the enzyme was applied to an RNA pool of a representative sample of conceptuses.

All primers were designed using Primer Blast software[1] (**Table 1**). In order to identify the most suitable housekeeping genes, duplicate qPCR assays were performed in a total volume of 20 μL, containing 10 μL Fast SYBR Green Master Mix (ThermoFisher Scientific), 1.2 μL forward and reverse primer mix (5 nM final concentration), 5.1 μL Nuclease-Free Water (ThermoFisher Scientific) and 2.5 ng of a representative sample of embryos. The Applied Biosystems 7500 Real-Time PCR Systems (ThermoFisher Scientific) was used and the thermo-cycling conditions were as follows: 1 cycle of holding stage at 50°C for 2 min and 95°C for 10 min; 40 cycles of cycling stage at 95°C for 15 s and 60°C for 1 min and, finally, 1 cycle of melt curve stage at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s. The presence of a single sharp peak in the melt curve analysis confirmed the specificity of all targets. A total of eight potential reference genes [Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*), Actin Cytoplasmic 1 (*ACTB*), 60S Ribosomal Protein L18 (*RPL18*), Peptidyl-Prolyl *Cis-Trans* Isomerase A (*PPIA*), 14-3-3 Protein Zeta/Delta (*YWHAZ*), RING Finger Protein 11 (*RNF11*), Histone H3.3 (*H3F3A*), Succinate Dehydrogenase Complex Subunit A Flavoprotein Variant (*SDHA*)] were analyzed using the geNorm function of the Qbase + package (Biogazelle; Zwijnaarde, Belgium) to identify the most appropriate for the study (Vandesompele et al., 2002). Because they were more stably expressed (average GeNorm $M \leq 0.5$), the reference genes selected were *RPL19* and *PPIA*.

Primer efficiency was carried out for the genes of interest, and qPCR of 1:4 dilutions of a cDNA mix from a representative pool of conceptuses were analyzed. The presence of a single sharp peak in the melt curve as well as the standard curve was used to confirm primer specificity. Average primer efficiency was 93.0 ± 4.7%. The expression of these genes was individually evaluated in 76 conceptuses (20 ovoid, 20 tubular, 36 filamentous) using 2.5 ng of cDNA, 10 μL of Fast SYBR™ Green Master Mix, 1.2 μL of 5 nM primers and 5.1 μL nuclease-free water, and the thermo-cycling conditions previously detailed.

The comparative Livak Ct method (ΔΔCt method; Livak and Schmittgen, 2001) was used to quantify the relative gene expression levels. First, for each conceptus, the expression of the gene of interest was normalized to the expression of the two housekeeping genes (*RPL19* and *PPIA*), using the following formula: $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{RPL19+PPIA}/2}$. To calculate the ΔΔCt, results were scaled to the average ΔCt across all conceptuses per target. The ΔCt values were used for the subsequent statistical analysis and results are presented as $2^{(-\Delta\Delta Ct)}$.

Statistical Analysis

Data relating to conceptus and CL sizes were checked for normality and homogeneity of variance by histograms, Qplots, and formal statistical tests as part of the UNIVARIATE procedure of SAS (version 9.1.3; SAS Institute, Cary, NC, United States). Conceptus size data were not normally distributed and, as such, were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box–Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate *P* values. The corresponding least squares means and standard error of the non-transformed data are presented in the results. Conceptus data and CL data (on Days 7 and 14) were analyzed using a mixed model (PROC MIXED of SAS). The model had experimental treatment (Control or Vasectomized) as a fixed effect, and heifer within treatment was included as a random effect. Differences between treatments were determined by *F* tests using type III sums of squares. The PDIFF command

[1] https://www.ncbi.nlm.nih.gov/tools/primer-blast/

incorporating the Tukey test was applied to evaluate pairwise comparisons between treatment means. Values were statistically significantly different when $P \leq 0.05$ and considered a tendency when $P \leq 0.10$.

Gene expression data were analyzed with IBM SPSS 25.0 for Windows (Armonk; New York, NY, United States). First, data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene test), premises for linear models. In those cases in which these premises were not met, data (x) were transformed using the arcsine of the square root (arcsin $\sqrt{x}$). Later, data (transformed or not depending on the case) were analyzed by an ANOVA of two factors followed by a Sidak *post hoc* test for pairs comparison. Since even after arcsin-transformation, expression of *CALM1*, *CASP3*, *CITED1*, *DLD*, and *TFGB3* did not match with parametric assumptions, Scheirer-Ray-Hare and Mann-Whitney tests were used as alternatives. In all cases, the significance level was established at $P \leq 0.05$.

RESULTS

Effect of SP Exposure on CL Size

Exposure to SP through mating with a vasectomized bull did not elicit differences in the CL volume at Day 7 or at Day 14 (Day 7: 7.1 ± 0.76 cm^3 vs. 6.5 ± 0.49 cm^3, for mated and unmated heifers, respectively, $P > 0.05$; Day 14: 3.1 ± 0.39 cm^3 vs. 4.5 ± 0.91 cm^3, respectively, $P > 0.05$). Similarly, no difference in CL weight on Day 14 was observed between SP-exposed and control heifers (5.1 ± 0.46 g vs. 6.4 ± 0.79 g, respectively; $P > 0.05$).

Effect of SP Exposure on Embryo Viability and Morphology

Conceptus recovery rate was similar from mated (exposed to SP) and unmated heifers (86/168: 51 ± 8.4% vs. 78/153: 51 ± 8.1%, respectively, $P > 0.05$; **Figure 2A**), indicating a lack of effect of SP-exposure on the survival of the transferred embryos. As is normal in cattle studies in which multiple-embryo transfer is carried out (O'Hara et al., 2014b), considerable variation in conceptus length within heifer was observed in both groups (CV 44–79%). However, conceptuses recovered from heifers mated to vasectomized bulls tended to be longer than those recovered from control heifers (16 ± 1.3 mm vs. 12 ± 1.2 mm, respectively, $P = 0.07$; **Figure 2B**).

Moreover, although no differences were observed in the percentage of ovoid, tubular and filamentous conceptuses recovered between groups (24/86: 26 ± 8.8%, 40/86: 40 ± 6.4%, 22/86: 18 ± 6.2% in control heifers and 21/78: 23 ± 6.2%, 30/78: 36 ± 6.9%, 27/78: 24 ± 6.8% in mated heifers; $P > 0.05$; **Figure 2C**), filamentous conceptuses recovered from SP-exposed heifers were longer than those recovered from control heifers (33 ± 2.2 mm vs. 27 ± 1.8 mm, respectively; $P < 0.05$; **Figure 2D**). Due to the large range in filamentous conceptus length, this group was subdivided into short filamentous (20–25 mm) and long filamentous (>25 mm) for subsequent gene expression analysis. In the control group, 6/86 conceptuses (7 ± 3.8%) were classified as long filamentous (average size 36 ± 4.7 mm; $n = 6$). In the SP-exposed group, 14/78 (18 ± 5.6%) conceptuses exhibited this morphology (average size 42 ± 2.4 mm; $n = 14$).

Effect of SP on Conceptus Gene Expression

In order to more accurately evaluate the developmental stage of the conceptuses, the relative abundance of transcripts for five candidate genes (*CALM1*, *CITED1*, *DLD*, *HNRNPDL*, and *TFGB3)* previously described as gastrulation markers (Degrelle et al., 2011) was assessed. However, the expression profiles described by Degrelle et al. (2011) in association with different developmental stages were not observed. Nevertheless, these genes are involved in important pathways for embryo development, and their expression changes are temporally regulated. In order to better characterize these pathways, an additional set of genes related to metabolism (*GSTM1* and *PTGS2*), apoptosis (*CASP3*), development (*FURIN*), and immunology (*JSP1* and *IL6*) (Mamo et al., 2011; Barnwell et al., 2016) was also interrogated. All these genes were analyzed in individual conceptuses exhibiting different morphologies (ovoid, tubular, short filamentous, and long filamentous).

The relative abundance of *CITED1, HNRNPDL, IL6, JSP1,* and *TGFB3* was affected by conceptus morphology in both the control and vasectomized groups. Long and short filamentous conceptuses recovered from control or SP-exposed heifers had lower *JSP1* expression ($P < 0.05$; **Figure 3I**). On the other hand, in both treatments, *IL6* relative expression was higher in short filamentous conceptuses in comparison to ovoid embryos ($P < 0.05$; **Figure 3H**); control long and short filamentous embryos also had higher relative abundances of this gene compared to ovoid and tubular conceptuses ($P < 0.05$; **Figure 3H**). In control conceptuses, *CITED1* and *TGFB3* relative expression was highest in long filamentous conceptuses ($P < 0.01$; **Figures 3C,K**), while in the vasectomized group such conceptuses exhibited the lowest relative expression of *CITED1* ($P < 0.05$; **Figure 3C**) and both long and short conceptuses had lower *TGFB3* expression compared to ovoid conceptuses ($P < 0.01$; **Figure 3K**). Additionally, *HNRNPDL* relative expression in the control group was highest in short filamentous conceptuses ($P < 0.01$; **Figure 3G**), whereas in the vasectomized group, the expression of this gene was lowest in both short and long filamentous conceptuses ($P < 0.01$; **Figure 3G**). In addition to these, in the control group, long filamentous conceptuses also exhibited the lowest *CALM1* and *DLD* relative abundance ($P < 0.01$; **Figures 3A,D**); while *FURIN* relative expression in short filamentous conceptuses was lower than ovoid and tubular embryos ($P < 0.05$; **Figure 3E**). On the contrary, higher *PTGS2* expression was detected in long and short filamentous conceptuses in comparison to ovoid conceptuses ($P < 0.05$; **Figure 3J**).

Relative expression of *CALM1, CITED1, DLD, HNRNPDL, PTGS2,* and *TGFB3* differed between treatments. Long filamentous conceptuses recovered from mated heifers presented lower *CITED1, HNRNPDL*, and *TGFB3* expression, and higher

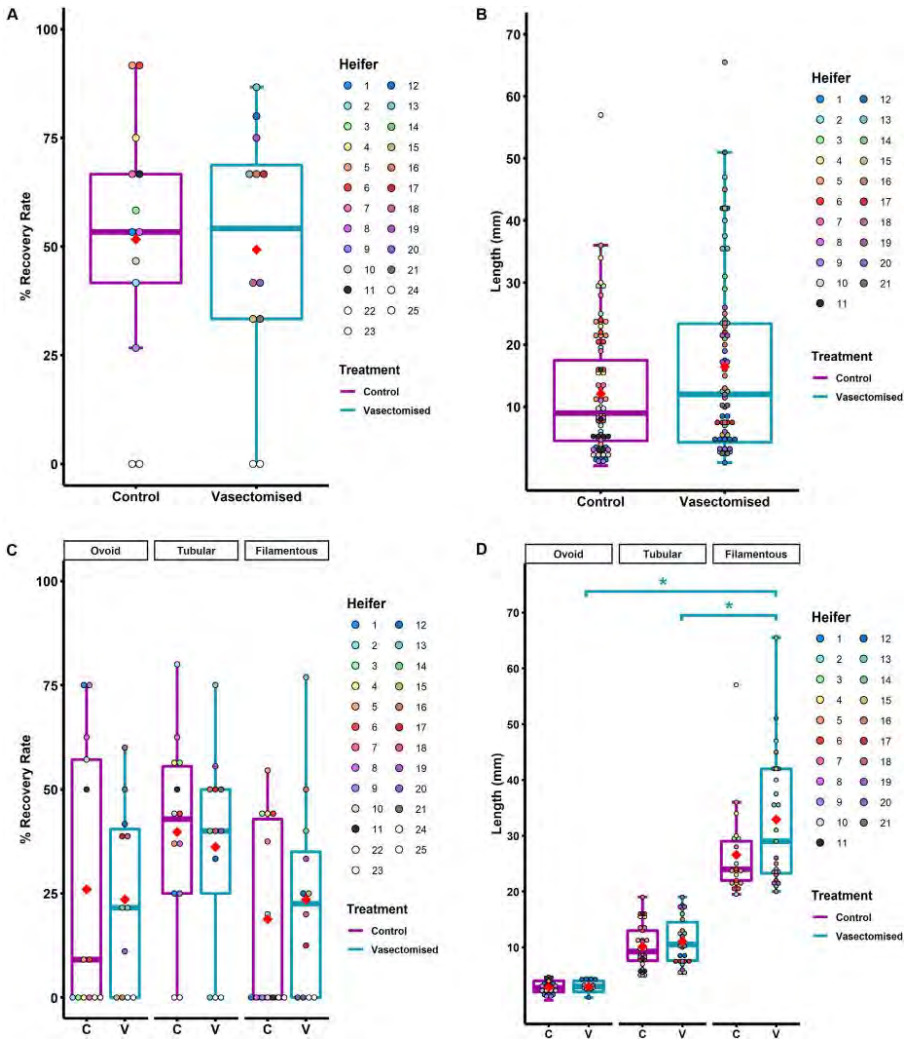

FIGURE 2 | (A) Conceptus recovery rate at Day 14 post-mating (vasectomized; *n* = 12 heifers) or post-estrus onset (control; *n* = 13 heifers). Each color represents one heifer. **(B)** Length of the recovered conceptuses post-mating (vasectomized; *n* = 78) or post-estrus onset (control; *n* = 86). Dots represent the conceptus length and their color reflects the heifer from which they were recovered. **(C)** Conceptus recovery rate at Day 14 depending on morphology (for control (C): ovoid *n* = 24, tubular *n* = 40, filamentous *n* = 22; for vasectomized (V): ovoid *n* = 21, tubular *n* = 21, filamentous *n* = 27). Dots represent the recovery rate of each heifer for each morphology. **(D)** Length of the recovered conceptuses depending on morphology [for control (C): ovoid *n* = 24, tubular *n* = 40, filamentous *n* = 22; for vasectomized (V): ovoid *n* = 21, tubular *n* = 21, filamentous *n* = 27]. Dots represent the conceptus length and their color depends on the heifer from which they were recovered. In all graphs, means are represented as red rhombus. Control group box-plots are represented in purple and vasectomized group box-plot, in blue. For the dots, light scale colors correspond to the control group and dark scale, to heifers mated to vasectomized bulls. Differences are indicated as $^{*}P \leq 0.05$.

CALM1 and *DLD* expression levels, compared to morphology-matched conceptuses recovered from control heifers ($P < 0.05$; **Figure 3**). In addition, *PTGS2* relative expression was higher in tubular conceptuses recovered from conceptuses that developed in a SP-primed environment than in control heifers ($P < 0.05$; **Figure 3J**).

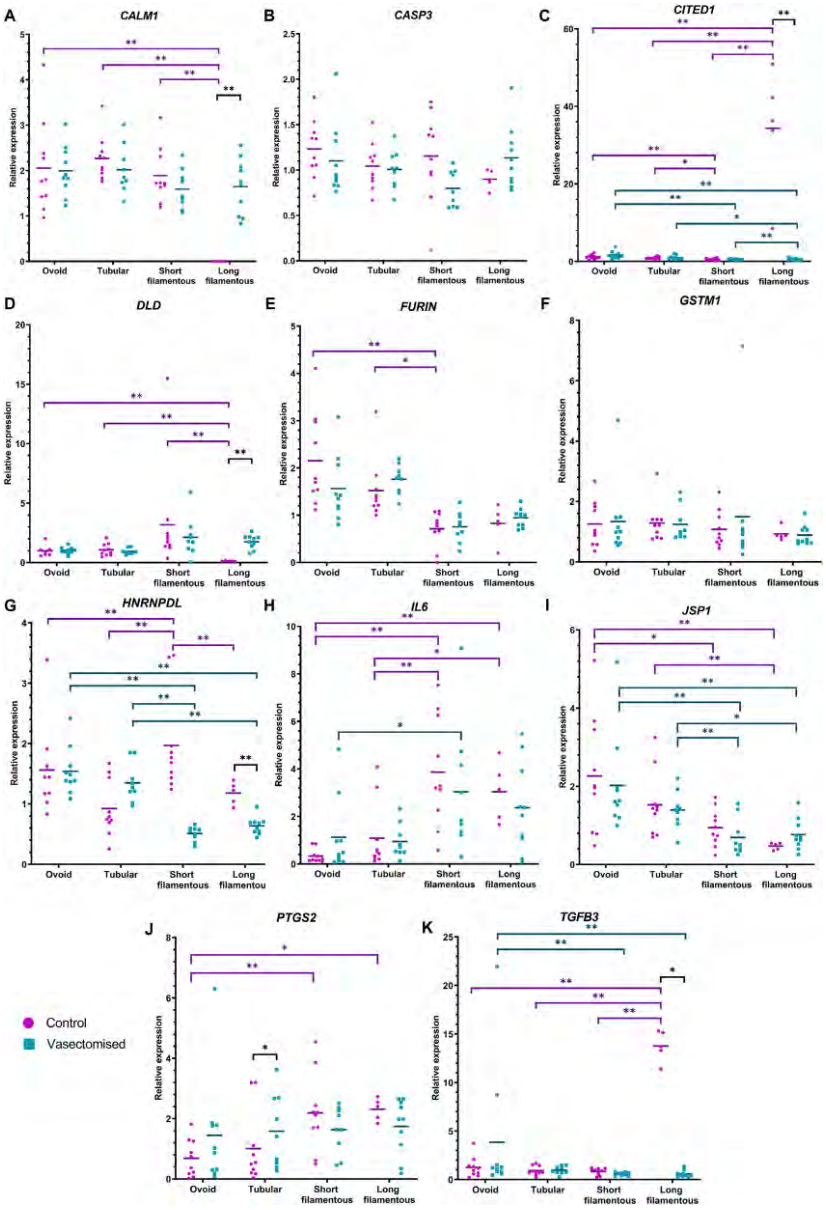

FIGURE 3 | Relative expression of *CALM1* **(A)**, *CASP3* **(B)**, *CITED1* **(C)**, *DLD* **(D)**, *FURIN* **(E)**, *GSTM1* **(F)**, *HNRNPDL* **(G)**, *IL6* **(H)**, *JSP1* **(I)**, *PTGS2* **(J)**, and *TGFB3* **(K)** among the different conceptus morphologies (for control: ovoid $n = 10$, tubular $n = 10$, short filamentous $n = 10$ and long filamentous $n = 5$; for vasectomized: ovoid $n = 10$, tubular $n = 10$, short filamentous $n = 10$ and long filamentous $n = 10$). Individual expression values (calculated with $2^{-\Delta\Delta Ct}$ method, using the housekeeping genes *RPL19* and *PPIA*) with bar representing the mean are presented. Purple dots correspond to the control group and blue squares correspond to the vasectomized group. Differences are indicated as $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

DISCUSSION

The main findings of this study are that exposure of heifers to SP through natural mating with vasectomized bulls: (1) does not elicit changes in the size of Day 7 or Day 14 CL; (2) does not improve embryo survival to Day 14, but (3) is associated with an increase in conceptus length and (4) alteration in expression of *CALM1*, *PTGS2*, *CITED1*, *DLD*, *HNRNPDL*, and *TGFB3*.

In recent years, the paternal influence on offspring health has gained increasing interest. This is due to studies showing that paternal health and nutrition can affect offspring development, and such effects can be carried over to the next generation (Morgan et al., 2020). One may immediately assume that the changes elicited in the embryo and subsequent individual are directly linked to abnormalities in the sperm of these males, which manage to fertilize the oocyte and transmit certain epigenetic signatures. However, mating of artificially-inseminated females to vasectomized mice fed different diets also has an effect on offspring outcomes (Watkins et al., 2018; Morgan et al., 2020), indicating that SP-induced changes in the female reproductive tract at the time of mating affect embryo development. Indeed, studies in mice and pigs have demonstrated that SP plays a role in the modulation of the maternal environment and, as a result, improves embryo survival and implantation (Johansson et al., 2004; O'Leary et al., 2004; Bromfield et al., 2014). In cattle, however, while SP infusion into the uterus alters the expression of certain genes (Ibrahim et al., 2019), this is not correlated with improved pregnancy rates (Odhiambo et al., 2009; Ortiz et al., 2019). These differences between species may reflect differences in SP composition (Rodger, 1976; Druart et al., 2013). As SP is a complex secretion produced by different accessory glands, variation in the type, structure and size of these organs can have a major impact on its composition (Bedford, 2015). For example, while the boar has large bulbourethral, prostate and vesicular glands, the latter two are relatively small in the bull (Druart et al., 2013). These two species only share 34% of their SP proteins in common (Druart et al., 2013). Perhaps more interesting to the subject at hand, both rodents and boars have an additional accessory gland that is lacking in the bull: the coagulating gland. This gland contributes to semen coagulation after ejaculation, which has been suggested to make sperm coating by SP proteins highly inefficient (Lefebvre et al., 2007). Thus, direct contact of the endometrium with the ejaculate might be more important in these species than in the bovine, where sperm can act as a vehicle for SP proteins. Indeed, it is important to note that in both mice and pigs, SP reaches the uterus during mating, while in cattle, the ejaculate is deposited in the anterior vagina (Hawk, 1983), and it is not clear whether any reaches the uterus without sperm involvement. Thus, models based on SP infusion directly into the uterus (Odhiambo et al., 2009; Ortiz et al., 2019) might not be representative of the events that take place physiologically. For this reason, in the current study, a model based on mating heifers to vasectomized bulls was used.

In addition to having an effect on the endometrium, studies in both mice and pigs have reported influences of SP on the ovary (Gangnuss et al., 2004; O'Leary et al., 2006). Exposure to SP (through mating in the mouse, or infusion into the uterus in the pig) led to an increase in macrophage recruitment into the ovulatory follicle (Gangnuss et al., 2004; O'Leary et al., 2006). In the pig, this was associated with an increase in CL weight at Days 5–9, which probably explains the increase in P4 secretion that was also observed at this time (O'Leary et al., 2006). Although there is inconsistent evidence on the effect of high P4 on embryo survival in pigs, with some authors reporting a positive effect (Ashworth, 1991; Jindal et al., 1997), others a negative (Mao and Foxcroft, 1998), and others a lack of effect (Muro et al., 2019), P4 prevents embryo resorption (Aisemberg et al., 2013) and is essential for timely progression of early embryogenesis (Zhang and Murphy, 2014) in mice. In cattle, elevated P4 concentrations prior to Day 7 are associated with an altered endometrial transcriptome (Forde et al., 2009) and accelerated conceptus development (Clemente et al., 2009; O'Hara et al., 2014a). Thus, increased P4 output could be one mechanism through which SP induces an increase

TABLE 1 | Primer design.

Gene	RefSeq (Bos taurus)	Forward primer	Reverse primer	Tm (°C)	Amplicon size (bp)
RPL19	NM_001040516.1	GAAAGGCAGGCATATGGGTA	TCATCCTCCTCATCCAGGTT	60	86
PPIA	NM_178320.2	CATACAGGTCCTGGCATCTTGTCC	CACGTGCTTGCCATCCAACC	60	108
CALM1	NM_001242572.1	GGATGGCAACGGGTACATCA	CTCCTCGTCCGTCAGCTTC	60	79
CASP3	NM_001077840.1	ACCAACGGACCCGTCAATTT	CCTCGGCAGGCCTGAATAAT	60	107
CITED1	NM_174518.1	TCACCTCCCACCAATTTATCCAA	TTGGCATTCTCCTTCACAGGT	60	110
DLD	NM_001206170.2	CGATGGCAGCACTCAAGTTA	CCTTGTTTTTGAAGGATACGTTG	60	306
FURIN	XM_024981598.1	GTTCGGCAACGTGCCCTG	TTCTTATTGGCCTCCAGGGTGAG	60	195
GSTM1	XM_010803234.3	GGACTTTCCCAATTTGCCCTAC	GCAATGTAGCGAAGGATGGC	60	78
HNRNPDL	XR_235028.4	GTGGCTATGGCGGCTATGAT	TGTTGGCCACTGTAGTCTGC	60	85
IL6	NM_173923.2	GCGCATGGTCGACAAAATCT	AAATCGCCTGATTGAACCCAGA	60	158
JSP1	XM_024983412.1	TTCCTCACCATGGGCATCATTG	ATCGTTATTCTGTTCCCGGCTG	60	172
PTGS2	NM_174445.2	CTGATGTTTGCATTCTTTGCCC	CTTAAGTCCACCCCATGGTTCT	60	107
TGFB3	NM_001101183.1	ACATAGCCAAGCAGCGGTAT	CCTAAGTTGGATTCTCTCCGCA	60	124

RefSeq corresponds to Gene NCBI accession number. PCR conditions: melting temperature in °C (Tm) and amplicon size in bp.

in embryo survival in this species. However, in the present study, no differences in CL volume at Days 7 and 14, nor in Day 14 CL weight were observed between treatments.

Exposure to SP at the time of mating had no effect on embryo survival to Day 14 following transfer on Day 7. Recovery rate is related to conceptus survival, as those who die degenerate and are not recovered on Day 14. This is consistent with the studies that indicate that SP infusion into the uterus does not lead to improved pregnancy rates in cattle (Odhiambo et al., 2009; Pfeiffer et al., 2012; Ortiz et al., 2019), and contrasts with some literature available in other species where exposure to SP leads to improved embryo survival and early embryo development (Johansson et al., 2004; O'Leary et al., 2004; Bromfield et al., 2014). Despite the lack of differences regarding embryo survival, filamentous conceptuses recovered from heifers that had been mated were longer than those recovered from control heifers. As already mentioned, large variation in conceptus size recovered from the same recipient is typically observed when multiple embryo transfer is carried out (O'Hara et al., 2014b; Barnwell et al., 2016) and is also seen after insemination in single ovulating cows (Ribeiro et al., 2016). Nevertheless, when conceptuses were grouped according to morphology (thus, reducing variation), filamentous conceptuses recovered from mated heifers were longer than their control counterparts. It is not clear whether an increase in conceptus length on as given day is a positive or negative phenomenon. On the one hand, a higher number of trophectoderm cells will ultimately lead to an increase in the secretion of IFNT, the maternal recognition signal in cattle. Maternal recognition of pregnancy in this species takes place around Day 16 (Sánchez et al., 2018). At this time, conceptuses that are not able to produce sufficient amounts of IFNT will be lost due to their inability to prevent luteolysis. On the other hand, asynchronous transfer of embryos, in which a Day 7 embryo is transferred to a Day 9, results in higher conceptus length but this does not translate into higher pregnancy rates (Randi et al., 2016).

The success of embryo transfer (in the absence of exposure of the reproductive tract of the recipient to either sperm or SP) in many livestock species, where pregnancy rates are comparable to those achieved with artificial insemination (Drost et al., 1999; Sartori et al., 2006), indicates that exposure to SP is not essential for pregnancy. However, as mentioned above, this factor does seem to have an impact on embryo and offspring metabolism and overall health. Having observed differences in conceptus length between embryos developing in an environment that had been exposed to SP or not, the next aim was to determine whether this difference in size was accompanied by a difference in development stage. Because morphology and size might not be representative of the developmental stage of the conceptus, a panel of genes previously reported to be markers of gastrulation stage (Degrelle et al., 2011) was evaluated. However, in the present study, the expression profiles described by Degrelle et al. (2011) were not observed, but we did detect differences in the expression of these genes between morphologies. In both groups, filamentous conceptuses exhibited the highest expression of *IL6*, and the lowest expression of *JSP1*. This expression pattern is consistent with prior studies, who detected upregulation of *IL6* and downregulation of *JSP1* in Day 15 bovine long conceptuses (measuring 24.7 $\pm$ 1.9) in comparison to short conceptuses (measuring 4.2 $\pm$ 0.1; Barnwell et al., 2016). On the other hand, in the present study, control filamentous conceptuses exhibited the highest *CITED1* relative expression, while the opposite was true in conceptuses recovered from mated heifers. In a study by Mamo et al. (2011), Days 16 and 19 bovine conceptuses had higher expression of *CITED2* (an important paralog of *CITED1*) than Days 7, 10 and 13 conceptuses, seemingly agreeing with our control group. *HNRNPDL* and *TGFB3* relative abundance also presented different pattern between conceptuses recovered from mated or unmated animals. While control filamentous embryos had the highest relative expression of both genes, the morphology-matched conceptuses in the vasectomized group has the lowest expression.

In addition, *CALM1*, *DLD*, and *FURIN* relative expression was lowest in filamentous conceptuses, whereas *PTGS2* was highest, only in the control group. In accordance with our results, *PTGS2* expression has previously been shown to be upregulated in Day 15 long conceptuses in comparison to age-matched short conceptuses (Barnwell et al., 2016), and in Days 16 and 19 in comparison to Days 7, 10, and 13 conceptuses (Mamo et al., 2011). However, *FURIN* expression was reported to be also upregulated in Days 16 and 19 in comparison to Days 7, 10, and 13 conceptuses (Mamo et al., 2011), in disagreement to our findings.

Although the pattern described by Degrelle et al. (2011) did not allow us to determine the gastrulation stage of our conceptuses, these marker genes are involved in different pathways important to embryo survival and development. Thus, the panel of genes was supplemented with additional ones in order to describe the effect of the SP-exposed environment on conceptus apoptosis, metabolism, development, and immunology. Most of the differences between treatments detected in gene expression were observed when comparing long filamentous conceptuses. Indeed, conceptuses exhibiting this morphology had different *CALM1*, *CITED1*, *DLD*, *HNRNPDL*, *PTGS2*, and *TGFB3* expression levels depending on whether they developed in a mated or unmated heifer. Interestingly, in the control group, expression levels of these genes differed between short and long conceptuses, whereas this behavior was not detected in the conceptuses from the vasectomized group. This hints at changes in the regulation of different pathways occurring in very large embryos, which is altered by a different uterine environment elicited by SP exposure.

The apoptosis process was evaluated by analyzing *HNRNPDL* and *CASP3* relative expression. Lower levels of *HNRNPDL* relative expression were observed in long filamentous embryos from the SP-exposed group than in the control. *HNRNPDL* encodes for the heterogeneous nuclear ribonucleoprotein D Like (hnRNPDL), a RNA-binding protein which binds heterogeneous nuclear RNA (hnRNA) to regulate pre-mRNA in the nucleus (Geuens et al., 2016). When hnRNPDL binds the specific mRNA, it induces the decay of the molecule (Fialcowitz et al., 2005) and, for this reason, it is considered to repress the gene expression of its targets. One of its potential targets is the Cell Division Cycle and Apoptosis Regulator 1 (*CCAR1*) (Li et al., 2019), which acts as a key intracellular transducer for apoptosis (Rishi et al., 2003). In addition, hnRNPDL also regulates the expression of

cytochrome C oxidase subunit 5B (*COX5B*), a mitochondrial energy-generating enzyme critical for the proper functioning of cells. The disruption of its expression by hnRNPDL may result in the cease of ATP generation and, therefore, the induction of apoptosis (Safavizadeh et al., 2012). Thus, a lower expression of *HNRNPDL* in the embryos could indicate lower levels of apoptosis. However, *CASP3*, a gene that codes for one of the proteases that initiates the execution pathway of apoptosis (intrinsic and the extrinsic apoptotic pathways diverge), did not differ between treatments. This suggests that SP-induced changes of *HNRNPDL* do not relate to apoptotic pathways in the conceptus and, therefore, its biological meaning remains unclear.

Conceptus metabolism was assessed by evaluating the relative expression of *DLD*, *GSTM1*, and *PTGS2*. *DLD* and *PTGS2* relative expression was higher in long filamentous or tubular embryos recovered from mated heifers than unmated heifers. *DLD* encodes for the mitochondrial dihydrolipoamide dehydrogenase, a member of the class-I pyridine nucleotide-disulfide oxidoreductase family crucial for embryo energy production (Leese, 1991; Johnson et al., 2009). Moreover, this enzyme seems to be essential for preimplantation embryos as *DLD* knockout mice embryos are unable to undergo gastrulation (Johnson et al., 1997). On the other hand, PTGS2 is the key enzyme in prostaglandin biosynthesis, which may mediate the effects of progesterone and IFNT in the endometrium and is highly expressed in the trophectoderm of ovine (Charpigny et al., 1997), bovine (Barnwell et al., 2016), porcine (Blomberg et al., 2006), and murine (Lim et al., 1999) embryos. The importance of this enzyme in embryo development is highlighted by the fact that *PTGS2* is downregulated in both *in vivo*- and *in vitro*-produced embryos that result in no pregnancy (El-Sayed et al., 2006; Ghanem et al., 2011). Considering both genes, it seems that SP may have an impact on the development in critical embryo stages such as tubular and filamentous embryos.

The relative expression of *CALM1*, *CITED1*, *FURIN*, and *TGFB3*, genes related to embryo development, were also evaluated. An effect of SP treatment in the long filamentous conceptuses for *CALM1*, *CITED1*, and *TGFB3* was observed. *CALM1* was the only one of these gene in which the relative expression increased in the long filamentous conceptuses recovered from mated heifers compared to control. On the other hand, both *CITED1* and *TGFB3* exhibited lower relative expression in conceptuses developing in a SP-primed environment in comparison to the control. *CALM1* encodes calmodulin 1, a calcium binding protein which represents the major calcium sensor in eukaryotes. *CALM1* has been associated to the morphogenesis process for the development of the body plan during gastrulation in response to global calcium waves (Slusarski and Pelegri, 2007), the early development of the neural system (Seto-Ohshima et al., 1987) and hematopoiesis (Kitsos et al., 2005). The participation of *TFGB3* in embryogenesis can be related to its role in the epithelial-mesenchymal transitions (EMT), which enables cell movement and morphogenesis (Zavadil and Böttinger, 2005). During gastrulation, EMT is observed in the generation of the primitive mesoderm, the cell migration into the primitive node and the establishment of the three embryonic layers (Blomberg et al., 2008; Dimitrova et al., 2017). *CITED1* (or *MSG1*), which encodes for the transcriptional factor Cbp/p300-interacting transactivator 1, has been described to be involved during embryogenesis (Dunwoodie et al., 1998; Gerstner and Landry, 2007) and placentation (Rodriguez et al., 2004; Sparrow et al., 2009) in mice. In summary, the lower expression of *CITED1* and *TGFB3*, together with higher levels of *CALM1*, suggest that long filamentous conceptus in the mated group may be at a later stage of gastrulation than morphology-matched control conceptuses. Finally, the relative expression of *IL6* and *MHC*-I (or *JSP1)*, two immune system related genes, was also evaluated, but no differences between treatments were observed.

To the best of our knowledge, this is the first study describing the effects of SP (as assessed by comparing unmated controls with heifers mated to vasectomized bulls) on the CL and early embryo development in cattle. The weight of evidence suggests that SP does not play a crucial role in embryo development in cattle as: (1) it is not clear whether SP reaches the uterus in bovine; (2) SP has been described to have a negative effect on endometrial RNA integrity *in vitro* (Fernandez-Fuertes et al., 2019); and (3) there is no evidence of an effect of SP exposure on pregnancy rates (Odhiambo et al., 2009; Pfeiffer et al., 2012; Ortiz et al., 2019). On the other hand, the embryo-related changes reported in the present work suggest that exposure to SP during natural mating changes the environment in which embryos develop from Day 7 onward. However, it is not clear whether these changes may be driven directly by the female reproductive tract or by an earlier CL maturation. Thus, further research should be conducted to elucidate the exact mechanism by which SP may improve embryo development.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College Dublin, Ireland, and the Universitat de Girona, Spain, and licensed by the Health Products Regulatory Authority (HPRA), Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes).

AUTHOR CONTRIBUTIONS

YM-O carried out the laboratory work, analyzed results and wrote the draft. JS, SR, SB-A, MM, and DK carried out the animal work, including handling of bulls and heifers, estrus detection, embryo transfer and recovery. MY performed statistical analysis of the data. MY, PL, JS, and BF-F contributed to the critical revision of the manuscript.

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Paper II

Aldose Reductase B1 in pig seminal plasma: identification, localization in reproductive tissues, and relationship with quality and sperm preservation.

Yentel Mateo-Otero, Estel Viñolas-Vergés, Marc Llavanera, Jordi Ribas-Maynou, Jordi Roca, Marc Yeste, Isabel Barranco.

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Aldose Reductase B1 in Pig Seminal Plasma: Identification, Localization in Reproductive Tissues, and Relationship With Quality and Sperm Preservation

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Aldose reductase B1 (AKR1B1), a NADPH-dependent enzyme that belongs to the aldo-keto reductase protein superfamily, has been reported to be involved in both male and female reproductive physiology. The objectives of this study were: (1) to evaluate the concentration of SP-AKR1B1 in pig ejaculate fractions; (2) to describe the immunohistochemical localization of AKR1B1 alongside the boar genital tract; (3) to evaluate the relationship between SP-AKR1B1 and sperm quality/functionality parameters. Ejaculates from seven boars (one ejaculate per boar) were collected in separate portions [the first 10 mL of the sperm rich fraction (SRF-P1), the rest of the SRF (SRF-P2), and the post-SRF (PSRF)], and the concentration of SP-AKR1B1 was assessed using an enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry and immunoblotting targeting was conducted in the reproductive tissues of these boars. Additionally, the entire ejaculates of 14 boars (one ejaculate per boar) were collected and split into three separate aliquots for: (i) SP-AKR1B1 quantification; (ii) assessment of sperm concentration and morphology; and (iii) evaluation of sperm quality and functionality parameters upon ejaculate collection (0 h) and after 72 h of liquid storage at 17°C. Concentration of AKR1B1 in the SP of SRF-P1 (458.2 ± 116.33 ng/mL) was lower ($P < 0.05$) than that of SRF-P2 (1105.0 ± 229.80 ng/mL) and PSRF (1342.4 ± 260.18 ng/mL). Monomeric and dimeric AKR1B1 forms were expressed alongside the reproductive tissues, except in the bulbourethral glands. No relationship between SP-AKR1B1 and sperm quality/functionality parameters was observed either at 0 h or after 72 h of storage at 17°C. In conclusion, AKR1B1 is expressed in the reproductive organs of boars (except bulbourethral glands) and a higher concentration is found in the PSRF suggesting that seminal vesicles would be the main secretory source. However, this enzyme does not appear to be related to sperm quality/functionality or to the sperm ability to withstand liquid storage at 17°C.

Keywords: aldose reductase B1, AKR1B1, seminal plasma, ejaculate fractions, sperm physiology, pig

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INTRODUCTION

Aldose reductase B1 (AKR1B1 or ALR2), a NADPH-dependent enzyme that belongs to the aldo-keto reductase protein superfamily (Bohren et al., 1989; Hyndman et al., 2003), has been reported to play an essential role in both male and female reproductive systems in humans (Bresson et al., 2011), cattle (Frenette et al., 2004; Girouard et al., 2009), rats (Kobayashi et al., 2002), sheep (Yang et al., 2019), and pigs (Steinhauser et al., 2016; Pérez-Patiño et al., 2018). This enzyme is known to be involved in the polyol pathway for fructose production, specifically in the conversion of glucose into sorbitol (Kobayashi et al., 2002). Interestingly, this pathway has been observed to occur during both epididymal sperm maturation in humans, cattle, and mice (Frenette et al., 2004, 2006; Jagoe et al., 2013) and conceptus peri-implantation period in pigs (Steinhauser et al., 2016). On the other hand, aldo-keto reductase enzymes have also been found to be implicated in catalyzing the reductive detoxification of carbonyl compounds within the genital tract of rat males (Kobayashi et al., 2002; Iuchi et al., 2004) and in prostaglandin 2 α synthesis (PGF2α) in pigs and humans (Bresson et al., 2011; Seo et al., 2014). As previous studies reported that AKR1B1 is expressed in the endometrium of humans (Bresson et al., 2011) and pigs (Seo et al., 2014) and is involved in PGF2α synthesis, it has been suggested that it could ultimately modulate conceptus implantation through regulation of endometrial gene expression in mammals (Kennedy et al., 2007; Seo et al., 2014). Finally, and reinforcing the belief that this protein plays a major role in reproduction, an in-depth proteomic analysis of pig seminal plasma (SP) revealed that AKR1B1 is positively related to *in vivo* fertility outcomes (Pérez-Patiño et al., 2018).

Seminal plasma, a complex fluid composed of secretions from the testis, epididymis, and male accessory sex glands, mixes with sperm upon ejaculation (Garner and Hafez, 2000). Although the classical roles attributed to SP are acting as a vehicle and serving as a nourishment media for sperm (Garner and Hafez, 2000), mounting evidence indicates that it plays a vital role for sperm function and modulates their fertilizing ability (Rodríguez-Martínez et al., 2011; Locatello et al., 2013). Moreover, SP also interacts with the female reproductive tract modulating the immune environment, a critical point required for successful pregnancy (O'Leary et al., 2004; Robertson, 2007; Schjenken and Robertson, 2014). For this reason, much research has focused on exploring the composition of SP, pointing to some SP-components as potential biomarkers of (in) fertility in several mammalian species (Novak et al., 2010; Milardi et al., 2013; Muhammad Aslam et al., 2014; Cazaux Velho et al., 2018; Pérez-Patiño et al., 2018; Leahy et al., 2019). In this regard, AKR1B1 in SP would be a clear candidate for a fertility biomarker due to its proven relationship with *in vivo* fertility (Pérez-Patiño et al., 2018). However, the mechanism through which SP-AKR1B1 could positively affect fertility in mammals remains unclear.

While the presence of AKR1B1 alongside the female reproductive system of several mammalian species has been extensively reported (Bresson et al., 2011; Seo et al., 2014; Steinhauser et al., 2016), the information about its role in the male genital tract and sperm physiology is scarce. Moreover, although AKR1B1 has been related to epididymal maturation in cattle (Frenette et al., 2004, 2006; Girouard et al., 2009) and mouse (Jagoe et al., 2013), the exact protein synthesis site in the male reproductive system is yet to be reported. In this sense, fractional emission of pig ejaculate offers a valuable opportunity to explore the contributions of specific male sex accessory glands to SP composition (Rodríguez-Martínez et al., 2009). Three fractions/portions can be objectively collected with clear differences in the SP source: (a) the first 10 mL of the so-called sperm rich fraction (SRF-P1), rich in sperm and with SP mainly originating from the epididymis; (b) the rest of SRF (SRF-P2), also rich in sperm and with SP mainly coming from the prostate (Rodríguez-Martínez et al., 2005, 2009), and, (c) the post-SRF (PSRF), poor in sperm and with SP mainly arising from the seminal vesicles (Einarsson, 1971; Rodríguez-Martínez et al., 2005, 2009; Saravia et al., 2009; Rodríguez-Martínez et al., 2011). Interestingly, the SP from these ejaculate portions has also been found to vary in terms of proteomic, metabolomic, and antioxidant capacity (Saravia et al., 2009; Rodríguez-Martínez et al., 2011; Barranco et al., 2015, 2016, 2017; Perez-Patiño et al., 2016; Mateo-Otero et al., 2020).

Considering the well-described relationship of SP-AKR1B1 with *in vivo* fertility outcomes in pigs (Pérez-Patiño et al., 2018) and the multiple key roles of AKR1B1 in reproductive physiology, the overall aim of the present study was to characterize the synthesis of SP-AKR1B1 alongside the male genital tract and to evaluate its involvement in sperm function. To this end, the specific objectives were: (1) to evaluate the concentration of SP-AKR1B1 in different pig ejaculate fractions; (2) to describe the immunohistochemical localization of AKR1B1 alongside the boar reproductive system; and (3) to evaluate the relationship between SP-AKR1B1 and sperm quality/functionality parameters in semen samples (sperm morphology, motility, viability, intracellular H_2O_2 production, acrosome integrity, and lipid disorder of plasma membrane). These sperm variables were assessed upon ejaculate collection and after 72 h of storage at 17°C, as liquid storage for that period of time is the most common method for preserving pig semen prior to conducting AI (Kumaresan et al., 2009; Waberski et al., 2019).

MATERIALS AND METHODS

Animals and Samples

All samples were supplied by an Artificial Insemination (AI) Center of AIM Ibérica located in Calasparra (Murcia, Spain), which fulfills the Spanish (ES300130640127; August 2006) and European (ES13RS04P; July 2012) legislation on commercialization of pig semen and animal health and welfare. Samples (ejaculates and reproductive tissues) were obtained from healthy and sexually mature boars (aged 18–36 months) from different breeds and crossbreeds (Pietrain and Duroc). Boars were housed individually in a building with controlled conditions of air temperature (15–25°C) and light (16 h per day), were fed with a commercial diet according to the nutritional requirements of adult boars (Chiba, 2009), and had *ad libitum* access to water.

Sperm quality of each ejaculate used in the experiment was assessed immediately after ejaculation following the standard procedures used in the AI center. All samples fulfilled the standards of sperm number and quality thresholds for the preparation of semen doses used for AI, specifically, (i) more than 200×10^6 spermatozoa/mL, (ii) more than 70% of motile sperm, and (iii) more than 75% of morphologically normal sperm.

Boars were slaughtered in a commercial slaughterhouse (La Mata de los Olmos, Teruel, Spain) for genetic replacement reasons while they were still healthy and suitable as semen donors. Once slaughtered, tissue samples (1 cm × 1 cm and 1 mm thick) from testis, epididymis, and accessory sex glands were collected. Fresh (for immunoblotting analysis) or fixed (4% phosphate-buffered formalin for immunohistochemical analysis) tissue samples were immediately frozen in liquid nitrogen.

Experimental Design

Experiment 1: SP-AKR1B1 Concentration in Ejaculate Portions

For the study of SP-AKR1B1 concentration in ejaculate fractions, the three fractions/portions (SRF-P1, SRF-P2, and PSRF) of seven ejaculates were collected separately, using the gloved hand method. The concentration of AKR1B1 in the SP of each portion was assessed using a porcine-specific enzyme-linked immunosorbent assay (ELISA), as described below.

Experiment 2: Expression of AKR1B1 in Male Reproductive Organs

Immunohistochemical and targeted immunoblotting analyses were conducted to find out which organs of the male reproductive system secreted AKR1B1. The samples, from three boars, came from medial testis; caput, corpus, and cauda segments of epididymis; and mid-areas of the prostate, seminal vesicles and bulbourethral glands.

Experiment 3: Relationship Between SP-AKR1B1 and Sperm Quality/Functionality Parameters

Entire ejaculates from 14 boars (one ejaculate per boar) were collected using a semi-automatic collection method (Collectis®, IMV Technologies, L'Aigle, France) and split into three aliquots. The first aliquot was used for sperm concentration and morphology assessment. The second one was extended alike AI-dose (30×10^6 sperm/mL in Biosem+ extender, Magapor, Zaragoza, Spain) and used to evaluate sperm quality and functionality parameters (sperm motility and viability, intracellular H_2O_2 production by viable sperm, and acrosome damage and plasma membrane lipid disorder of viable sperm) after ejaculate collection (0 h) and after 72 h of liquid storage at 17°C. Finally, the third aliquot was centrifuged to obtain SP. Next, SP samples were stored at −80°C until the concentration of AKR1B1 was analyzed with an ELISA assay.

Seminal Plasma Processing and Storage

Immediately after ejaculate collection, semen samples were centrifuged twice at 1,500 *g* and room temperature for 10 min (Rotofix 32A, Hettich Centrifuges UK, Newport Pagnell, Buckinghamshire, United Kingdom), following a previously described protocol (Perez-Patiño et al., 2016; Li et al., 2018; Barranco et al., 2019; Padilla et al., 2020). After the second centrifugation cycle, the supernatant was examined under a microscope (Eclipse E400, Nikon, Melville, NY, United States) to verify that it was sperm-free. Then, SP samples were split into cryotubes and stored at −80°C (Ultra Low Freezer, Haier Inc., Qingdao, China) until analysis. Samples were thawed on ice prior to evaluation.

Immunoblotting

In order to lysate tissue samples, 50 mg of each tissue was resuspended in 800 μL of lysis buffer (xTractor® Buffer; Takara Bio, Mountain View, CA, United States) supplemented with 50 U DNase I (Takara Bio), 1% protease inhibitor cocktail, and 700 mM sodium orthovanadate. After incubation at 4°C for 10 min, samples were disrupted mechanically four times using a TissueLyzer II (Qiagen, Hilden, Germany) set at 30 strokes/s for 5 min at 4°C. Subsequently, centrifugation at 12,000 *g* and 4°C for 30 min was carried out in order to obtain the supernatants, which were finally collected and stored at −80°C. Finally, total protein was quantified in triplicate by a detergent compatible (DC) method (BioRad).

For each tissue sample, 10 μg of total protein was diluted in 10 μL of miliQ water. Then, 10 μL of Laemmli reducing buffer 2× supplemented with 5% (v/v) β-mercaptoethanol (BioRad) was added to samples and boiled at 95°C for 5 min. Following this, a total volume of 20 μL per sample was loaded onto a polyacrylamide gradient (8–16%) gel (Mini-PROTEAN® TGX Stain-FreeTM Precast Gels, Bio-Rad); electrophoresis ran at 120–150 V for approximately 1 h. After electrophoresis, total protein content was visualized by UV exposition and acquisition using a G:BOX Chemi XL system (SynGene, Frederick, MD, United States). Afterward, proteins from the resulting gels were transferred onto polyvinylidene difluoride (PVDF) membranes using Trans-Blot® Turbo™ (BioRad). Next, membranes were blocked in blocking buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween-20, and 5% bovine serum albumin; pH = 7.3) (Roche Diagnostics, S.L., Basel, Switzerland) at room temperature for 1 h under agitation. One of the blocked membranes was incubated with the anti-AKR1B1 primary antibody (ref. HPA026425, Prestige Antibodies; Merck, Germany) diluted in blocking solution (1:2,000; v:v), and the other membrane with the same primary antibody (1:2000, v:v) and its blocking peptide (ref. APREST77862, Prestige Antibodies; Merck) 20 times more concentrated than the antibody. Both membranes were incubated at 4°C overnight. Next, membranes were washed three times with TBS-Tween 20 1× (10 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20; pH = 7.3) for 5 min before incubation with an anti-rabbit, secondary antibody conjugated with HRP (ref. P0448; Sigma Aldrich) diluted in blocking solution (1:3,000; v:v). Membranes were washed 10 times and finally revealed using a chemiluminescent substrate (Immobilion™ Western Detection Reagents, Millipore); images were scanned with G:BOX Chemi XL 1.4.

Immunohistochemistry

The sections of paraffin-embedded tissue samples (male reproductive organs and liver as a positive control) were immunohistochemically stained using an avidin-biotin complex protocol (Vector Laboratories, Burlingame, CA, United States). Briefly, sections were first deparaffinized two times using Histo-Clear II (Electron Microscopy Sciences, Hatfield, England) and progressively rehydrated through a decreasing ethanol series from 100 to 70% and distilled water. Next, and in order to allow antigen retrieval, sections were microwaved four times in 10 mM Tris-1 mM EDTA buffer (pH = 9.0) for 5 min, with intermediate refiling with Tris-EDTA. Then, sections were washed with tap water and placed in racks. Samples were incubated with a blocking and permeabilizing solution composed of 3% bovine serum albumin (BSA) in 0.1% PBS-Tween at room temperature for 30 min. Afterward, sections were incubated overnight at 4°C with the rabbit anti-AKR1B1 primary antibody diluted 1:100 (v:v) in PBS with 1% Triton X-100 containing 3% BSA. As a negative control, the primary antibody was omitted. On the other hand, in order to prove antibody specificity, samples were incubated with the AKR1B1 primary antibody and its blocking peptide, which was 50 times in excess. The next day, sections were washed and subsequently incubated with a polyclonal goat anti-rabbit secondary antibody conjugated with biotin (EDM Millipore Corporation, Temecula, CA, United States) diluted 1:200 (v:v) in PBS containing 1% Triton X-100 and 3% BSA at room temperature for 30 min. Sections were then washed and incubated with 3% H_2O_2 in BSA-PBS for 20 min to block endogenous peroxidase activity. Next, all sections were incubated with the VECTASTAIN ABC reagent (Vector Laboratories, Burlingame, CA, United States) for 1 h, and with DAB peroxidase substrate working solution (Vector Laboratories, Burlingame, CA, United States) for 10 min. Slides were counterstained with Harris hematoxylin (Thermo Fisher Scientific, Waltham, MA, United States), dehydrated with an increasing ethanol series, and mounted with Eukitt® mounting medium (PanReac, Barcelona, Spain). Finally, slides were microscopically evaluated and photographed using Nikon Eclipse EP2000-S (Nikon).

Enzyme-Linked Immunosorbent Assay

Concentration of AKR1B1 in SP was quantified using a porcine-specific quantitative sandwich ELISA kit (MBS9316209; MyBioSource, San Diego, CA, United States) following the manufacturer's manual. Briefly, to obtain the standard curve, 50 μL of AKR1B1 standards (0.625, 1.25, 2.5, 5, 10, and 20 ng/mL) was added to the plate in duplicate. On the other hand, SP samples were thawed, diluted in PBS 1× (1:75; v:v) and added to the plate in duplicate. The content of all wells, except the blank ones, was mixed with 100 μL of HRP-Conjugate Reagent, and the plate was subsequently incubated at 37°C for 60 min. After washing all wells four times, 50 μL of Chromogen A and 50 μL of Chromogen B were added. After mixing gently, the plate, protected from light, was incubated at 37°C for 15 min. Next, 50 μL of the Stop solution was added to all wells and, after 5 min, the plate was read at 450 nm using a microplate spectrophotometer (BioTek Epoch; BioTek, Winooski, Vermont, United States). The average of the duplicate reading for each standard was calculated and the average optical density from the blank was subtracted. Based on AKR1B1 standards, a linear regression curve interpolating AKR1B1 concentration from absorbance reading was calculated; the equation resulted to be: [AKR1B1] = Abs+0.038/0.0522, $R^2 = 0.9772$.

This ELISA kit was highly specific for porcine AKR1B1, showing a sensitivity of 0.1 ng/mL and a detection range of 0.625–20 ng/mL.

Sperm Quality and Functionality Parameters' Assessments

For evaluation of sperm quantity and functionality, seven parameters were assessed: (i) concentration, (ii) morphology, (iii) motility, (iv) viability, (v) intracellular H_2O_2 production by viable sperm, (vi) acrosome damage in viable sperm, and (vii) plasma membrane lipid disorder in viable sperm. Except for sperm concentration, which was only evaluated immediately after ejaculate collection (0 h), the other quality/functionality variables were determined at two time-points: after ejaculate collection (0 h) and after 72 h of liquid storage at 17°C (72 h).

For sperm concentration assessment, a high-precision automated cell counter (NucleoCounter®NC-100™, ChemoMetec, Allerod, Denmark) was used following manufacturer's recommendations. Sperm morphology was examined under a phase-contrast microscope at 1,000× magnification (Nikon Labophot, Nikon, Tokio, Japan) in semen samples diluted (1:1; v:v) with 0.12% formaldehyde saline solution (Panreac). A total of 200 sperm cells were counted and classified as morphologically normal if they did not exhibit abnormal head, acrosome defects, proximal cytoplasmic droplets, distal cytoplasmic droplets, folded tails, or coiled tails. Sperm motility was assessed using a computer-assisted sperm analyzer (CASA, ISASV1®, Proiser R+D S.L., Paterna, Spain). For this analysis, 5 μL of each semen sample (30×10^6 sperm/mL in Biosem+) was pipetted onto a pre-warmed (38°C) Makler chamber (Sefi Medical Instruments, Haifa, Israel). A total of 10 different fields per sample accumulating ≥600 sperm were acquired and examined. For further analysis, percentages of total motile sperm (sperm showing an average path velocity ≥20 μm/s) and progressively motile sperm (exhibiting rapid and progressive movement with a straight-line velocity ≥40 μm/s) were recorded.

Sperm viability, acrosome damage, intracellular H_2O_2 production, and membrane lipid disorder were assessed by flow cytometry (BD FACS Canto II; Becton Dickinson & Company, Franklin Lakes, NJ, United States). For each semen sample and sperm parameter, three technical replicates with 10,000 Hoechst 33342 (H-42; Merck)-positive events were evaluated.

Sperm viability and acrosome damage were assessed using a triple-staining with Hoechst 33342 (H-42), propidium iodide (PI; Merck), and fluorescein-conjugated peanut agglutinin

(PNA-FITC; Merck). Briefly, 100 μL of each semen sample (30×10^6 sperm/mL in Biosem+) was incubated with 3 μL H-42 (0.05 mg/mL in PBS), 2 μL PI (0.5 mg/mL in PBS), and 2 μL PNA-FITC (100 μg/mL in PBS) at 37°C (Sanyo MIR-153 incubator, Gemini BV, Apeldoorn, Netherlands) for 10 min. Next, 400 μL PBS was added to each sample. Percentages of viable spermatozoa (H-42+/PI-) with an intact (PNA-FITC-) and non-intact (PNA-FITC+) acrosome membrane were recorded.

To assess intracellular H_2O_2 production by viable sperm, a triple-staining with H-42, PI, and 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Merck) was prepared. Briefly, 50 μL of each semen sample (30×10^6 sperm/mL in Biosem+) was incubated with 1.5 μL H-42 (0.05 mg/mL in PBS 1×), 1 μL PI (0.5 mg/mL in PBS), and 1 μL CM-H_2DCFDA (1 mM in dymetilsulfoxide [DMSO]) in 950 μL PBS at 37°C for 30 min. An aliquot of each semen sample was incubated with all fluorochromes plus 1 μL of tert-butyl hydroperoxide solution (70% in distilled water) and was used as a positive control. The percentage of viable sperm (H-42+/PI−) that exhibited high intracellular H_2O_2 generation (2′,7′-di-chlorofluorescein [DCF]+) was recorded.

Finally, to evaluate the lipid disorder of plasma membrane in viable sperm, a triple-staining with H-42, Yo-Pro-1 (Merck), and Merocyanine 540 (M-540; Merck) was carried out. Briefly, 50 μL of each semen sample (30×10^6 sperm/mL in Biosem+) was incubated with 2.5 μL H-42 and 10 μL Yo-Pro-1 (2.5 μM in DMSO) in 950 μL PBS at 37°C for 8 min. After this period, 26 μL of M540 (0.1 mM in DMSO) was added to each sample and incubated at 37°C for 2 min. The percentage of viable spermatozoa (H-42+/Yo-Pro-1-) exhibiting lipid membrane disorder (M-540+) was recorded.

Statistical Analysis

Data were analyzed using the statistical package IBM SPSS 25.0 for Windows (IBM corp., Armonk, NY, United States). First, normal distribution was tested with Shapiro-Wilk test and homogeneity of variances was checked with Levene test. Concentrations of AKR1B1 between the three SP portions (i.e., SRF-P1, SRF-P2, and PSRF) were compared through one-way analysis of variance (ANOVA) followed by *post hoc* Sidak test. Ejaculates were classified based on their AKR1B1 concentration into groups through a two-step cluster analysis using the log-likelihood distance and the Schwarz's Bayesian criterion. Following this, sperm quality and functionality variables (sperm motility and viability, intracellular H_2O_2 production by viable sperm, acrosome damage in viable sperm, and plasma membrane lipid disorder in viable sperm) were compared between the two groups of ejaculates (high SP-AKR1B1 and low AKR1B1) with a linear mixed model followed by *post hoc* Sidak test. In this model, between-subjects factor was the ejaculate group and within-subjects factor was the time of semen storage at 17°C. When needed, data were linearly transformed with arcsine $\sqrt{x}$. The level of statistical significance was set at $P \leq 0.05$.

RESULTS

Experiment 1: SP-AKR1B1 Concentration in Ejaculate Portions

Concentrations of AKR1B1 in SP fractions/portions are shown in **Figure 1**. The SP from SRF-P1 from SRF-P1 exhibited the lowest ($P < 0.05$) AKR1B1 concentration (458.2 ± 116.33 ng/mL) compared to SRF-P2 (1105.0 ± 229.80 ng/mL) or PSRF (1342.4 ± 260.18 ng/mL). Moreover, no differences ($P > 0.05$) in AKR1B1 concentrations were found between SRF-P2 and PSRF. Finally, no breed effect was observed in SP-AKR1B1 concentration, as Pietrain and Duroc boars showed similar concentrations (522.2 ± 66.85 ng/mL vs. 691.7 ± 81.00 ng/mL; $P > 0.05$).

Experiment 2: Expression of AKR1B1 in Male Reproductive Organs

The immunoblotting assay revealed the presence of AKR1B1 along the entire male reproductive tissues except for bulbourethral glands (**Figure 2**). Specifically, two specific bands were detected: (i) a 36 kDa band was found in testis, epididymal caput and corpus, and seminal vesicles; and (ii) a ∼80 kDa band was also identified in testis, cauda, caput and corpus of epididymis, seminal vesicles, and prostate. Both bands also appeared in the positive control (liver). The two bands were not seen when membranes were incubated with AKR1B1 blocking peptide, revealing that they were specific for AKR1B1. Therefore, bands of ∼40 and ∼70 kDa should be considered as unspecific for this antibody.

The AKR1B1 protein was immunohistochemically detected in the reproductive tissues analyzed (**Figure 3**). Two controls

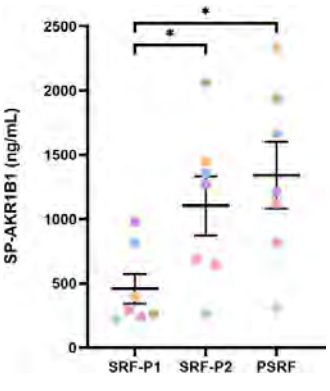

FIGURE 1 | Seminal plasma-AKR1B1 concentration (ng/mL) in the different pig ejaculate fractions: the first 10 mL of the sperm rich fraction (SRF-P1), the rest of the sperm rich fraction (SRF-P2), and post sperm rich fraction (PSRF). Data are represented as mean ± standard error of the mean. Different dot colors represent each individual ($n = 7$). Differences between fractions are indicated as * ($P \leq 0.05$).

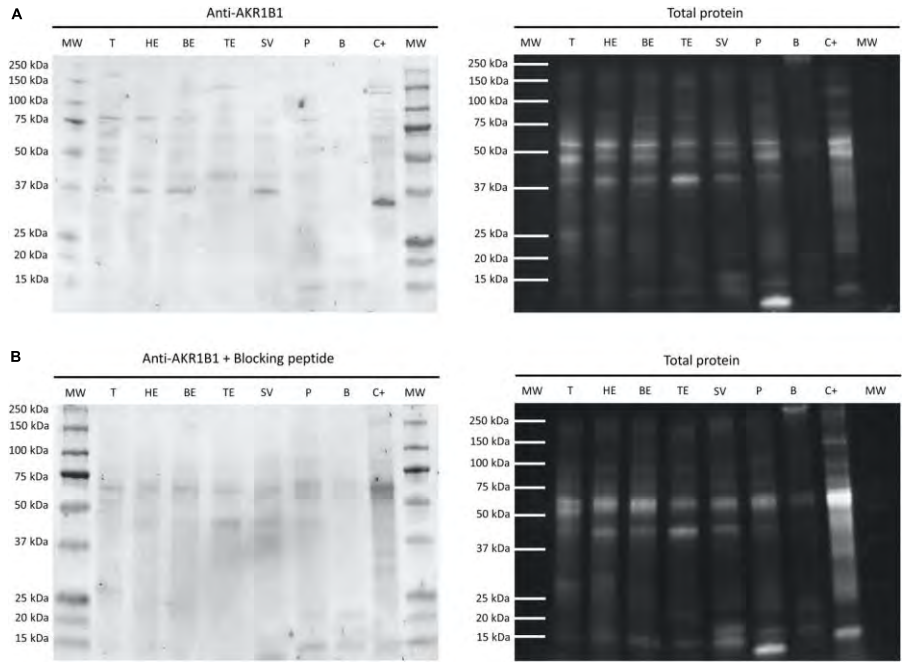

FIGURE 2 | Representative Western blot of **(A)** anti-AKR1B1 and **(B)** its corresponding blocking peptide, and their total protein controls in boar reproductive tissues. MW, molecular weight; T, testis; HE, caput epididymis; BE, corpus epididymis; TE, cauda epididymis; SV, seminal vesicles; P, prostate; B, bulbourethral glands; C+, liver.

were used: (i) the specificity of the primary antibody was confirmed in all tissues through incubation with the AKR1B1 primary antibody with blocking peptide and (ii) the specificity of the secondary antibody was proven with the omission of the primary antibody in all tissue samples. The presence of AKR1B1 was confirmed in all reproductive tissues except for bulbourethral glands, in which no staining was observed. In testis, AKR1B1 was observed in the interstitial tissue, specifically in the cytosol of Leydig cells, whereas no presence of the protein was detected in seminiferous tubules. Regarding epididymis, AKR1B1 was localized in basal and principal cells of the epithelia of all regions (caput, corpus, and cauda). In addition, both prostate and seminal vesicles showed cytoplasm immunostaining in glandular epithelial cells. Finally, AKR1B1 was undetectable in bulbourethral glands.

Experiment 3: Relationship Between SP-AKR1B1 and Sperm Quality and Functionality Parameters

In order to evaluate the relationship between SP-AKR1B1 and sperm quality and functionality parameters, 14 ejaculates were classified (hierarchical clustering; $P < 0.001$) into two groups: with low SP-AKR1B1 (ranging from 376.4 to 756.3 ng/mL, $n = 7$) and high SP-AKR1B1 levels (ranging from 842.2 to 1211.25 ng/mL, $n = 7$; **Figure 4**). No differences ($P > 0.05$) in any of the different sperm quality and functionality parameters assessed (sperm concentration, normal sperm morphology, total and progressive motility, viable sperm, viable sperm with high intracellular H_2O_2, viable sperm with a damaged acrosome, and viable sperm with high membrane destabilization) were observed between high and low SP-AKR1B1 groups at any evaluation time-point (0 and 72 h of storage at 17°C).

DISCUSSION

To the best of our knowledge, this is the first study characterizing the expression of AKR1B1 along the male reproductive system in livestock. Likewise, this report is also the first relating the concentration of AKR1B1 in SP with sperm quality and functionality parameters of liquid-stored semen samples. Specifically, the results showed that: (i) monomeric and dimeric AKR1B1 forms were expressed in all male reproductive tissues, except bulbourethral glands; (ii) AKR1B1 was expressed in Sertoli cells, basal and principal

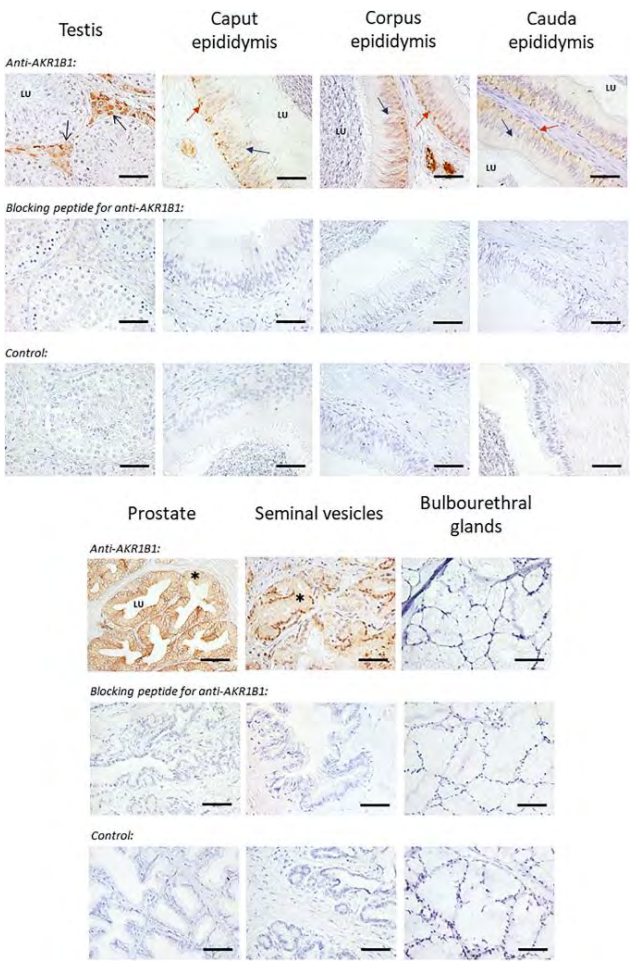

FIGURE 3 | Immunohistochemistry of AKR1B1 in boar genital organs (testis, caput, corpus, and cauda from the epididymis, prostate, seminal vesicles, and bulbourethral glands). The first row shows anti-AKR1B1 staining in each tissue. Specifically, Sertoli cells from testis (black arrow), principal (blue arrow), and basal cells (orange arrow) in the epididymis and glandular cells from prostate and seminal vesicles (indicated as *) appeared stained. Specificity of the primary antibody in all tissues/organs was confirmed by the absence of signal in blocking peptide assays (second row). Finally, negative control (third row) proved the specificity of the secondary antibody. LU, lumen. Scale bar = 50 μm.

epididymal cells, and glandular cells from the prostate and seminal vesicles; (iii) seminal vesicles were likely to contribute the most to the final SP-AKR1B1 concentration of the pig ejaculate; and (iv) SP-AKR1B1 levels were not related to sperm quality and functionality parameters, nor was this enzyme involved in the sperm resilience to preservation at 17°C for 72 h.

Characterization of SP components, including proteins or even metabolites, has been a source of sperm quality, functionality, and fertility biomarkers in several mammalian species (Milardi et al., 2013; Cazaux Velho et al., 2018; Moura et al., 2018; Pérez-Patiño et al., 2018). In this sense, as mentioned before, AKR1B1 has been reported to have multiple roles in the reproductive physiology of humans (Bresson et al., 2011), cattle (Frenette et al., 2004; Girouard et al., 2009), rodents (Kobayashi et al., 2002), and pigs (Steinhauser et al., 2016; Pérez-Patiño et al., 2018). However, no information regarding its synthesis along the male reproductive tract exists in any mammalian species.

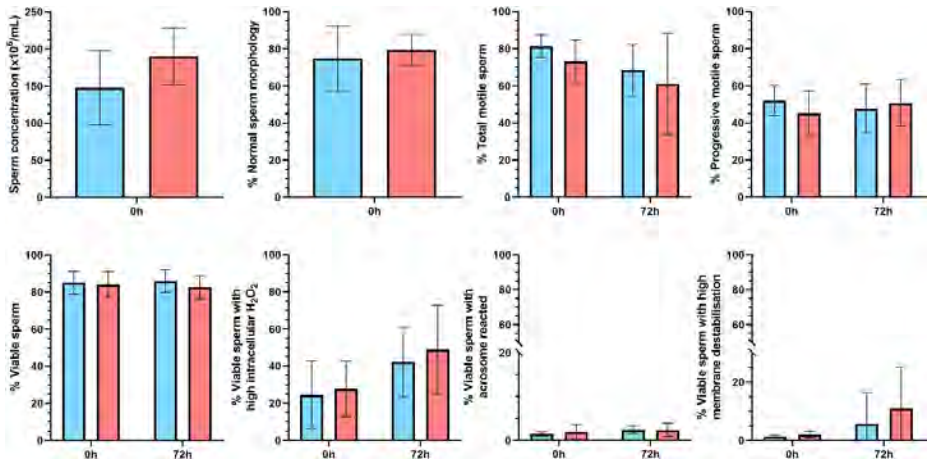

FIGURE 4 | Sperm quality and functionality parameters upon semen collection (0 h) and after 72 h of liquid storage at 17°C in two groups ejaculates clustered on the basis of AKR1B1 concentration in seminal plasma (SP) samples. Fourteen ejaculates were classified into two groups: with low SP-AKR1B1 levels (ranging from 376.4 to 756.3 ng/mL, *n* = 7; blue bars) and with high SP-AKR1B1 levels (ranging from 842.2 to 1211.25 ng/mL, *n* = 7; red bars). Data are represented as mean ± standard error of the mean.

The immunoblotting and immunohistochemistry results showed that AKR1B1 was expressed in testis, epididymis, and all accessory sex glands, except the bulbourethral ones. These results are in agreement with observations in male rats, in which the activity of aldose reductase was observed along all the male reproductive tract, except in bulbourethral glands that were not analyzed (Kobayashi et al., 2002; Iuchi et al., 2004). Moreover, other studies conducted in cattle and pigs demonstrated the presence of AKR1B1 in seminal vesicles (Samuels et al., 1962; Westfalewicz et al., 2017). This protein has been reported to interact with epididymal sperm during maturation (Frenette et al., 2003, 2006; Katoh et al., 2014), which would advise a role of AKR1B1 in sperm physiology. In this context, the presence of AKR1B1 has been proposed to contribute to the acquisition of sperm motility and fertilizing ability in pigs (Katoh et al., 2014), as well as to support bovine sperm survival during epididymal transit and storage (Frenette et al., 2003). In addition, our immunoblotting assay showed the presence of one or two specific bands (36 and ∼80 kDa) in most male reproductive tissues. While dimerization of AKR1B1 has been previously reported in ovine thymus (Yang et al., 2019) and bovine peripheral blood mononuclear cells (Yang et al., 2016), there are no previous studies of such a dimerization in SP samples from any mammalian species. Indeed, as monomeric and dimeric protein forms are known to have different cellular functions, further studies should address whether these forms could explain the different roles of SP-AKR1B1 in reproductive physiology.

Besides the presence of AKR1B1 along the male reproductive tract, our study found that this protein was only present in specific cell types, rather than in the lumen of the different organs. Specifically, in testis, only Leydig cells were stained with the anti-AKR1B1 antibody. Since these cells are implicated in hormonal secretion and spermatogenesis regulation (Zhou et al., 2019), one could hypothesize that AKR1B1 is involved in these processes. On the other hand, AKR1B1 was also found in both basal and principal cells along the entire epididymis and glandular cells from the prostate and seminal vesicles. Considering that all of these cell types are involved in protein secretion (Leung et al., 2004; Breton et al., 2019), the presence of AKR1B1 in SP can be assumed to be originated from the collective synthesis and secretion of the aforementioned organs. These findings are in agreement with a previous study conducted in bovine, in which aldose reductase was also reported to be present in the same epididymal and testicular cells lines (Frenette et al., 2003). However, this is the first report in a mammalian species describing that vesicle glands are the main synthesis site. Nevertheless, to the best of our knowledge, neither the exact contribution of the different male accessory glands to AKR1B1 levels in SP, nor its potential role on ejaculated sperm has been uncovered.

ELISA assay of the different ejaculate portions confirmed immunohistochemical and immunoblotting results, indicating that whereas AKR1B1 was present in the SP from all ejaculate portions, a higher concentration of this protein was found in the SP from PSRF. As aforementioned, SP from each ejaculate portion has a different origin: SP from SRF-P1 is mainly secreted by the epididymis, SP from SRF-P2 originates from the epididymis and prostate, and SP from PSRF is mainly produced by seminal vesicles (Einarsson, 1971; Rodríguez-Martínez et al., 2009; Saravia et al., 2009; Rodríguez-Martínez et al., 2011). Considering these results, one could hypothesize that seminal vesicles are the principal contributor for SP-AKR1B1 secretion to

the entire ejaculate. Similar results have been reported in bovine, where this enzyme is one of the most abundant proteins of the seminal vesicle fluid (Westfalewicz et al., 2017).

The present study also evaluated the relationship between SP-AKR1B1 concentration and sperm quality and functionality after both 0 and 72 h of liquid storage at 17°C. No relationship between SP-AKR1B1 concentration and any of the evaluated parameters, which included sperm concentration, normal sperm morphology, total and progressive motility, sperm viability, and percentages of viable sperm with high intracellular ROS, viable sperm with a damaged acrosome, and viable sperm with high membrane destabilization, was observed. To the best of our knowledge, there is scarce information regarding the role of AKR1B1 in sperm physiology. While AKR1B1 has been reported to be overexpressed in men with high seminal lipid peroxidation levels (Intasqui et al., 2015) and to be involved in porcine sperm capacitation (Katoh et al., 2014), none of these parameters were evaluated in the present study. Thus, taking into account the lack of influence of SP-AKR1B1 on sperm quality and functionality found in our study, together with the fact that the highest SP-AKR1B1 concentration was not found in the SRF (which contains most of the ejaculated sperm; Einarsson, 1971; Rodríguez-Martínez et al., 2009; Saravia et al., 2009; Rodríguez-Martínez et al., 2011), it is reasonable to surmise that SP-AKR1B1 does not play a major role on the sperm quality and functionality parameters assessed. However, this protein could play a crucial role on sperm lipid peroxidation or during capacitation. Further studies are required to evaluate this potential relationship.

Besides its relevance during epididymal maturation (Frenette et al., 2003; Katoh et al., 2014), SP-AKR1B1 could have a potential influence on the female reproductive tract, as it is expressed in the endometrium in humans (Chapdelaine et al., 2006; Bresson et al., 2011) and pigs (Seo et al., 2014; Steinhauser et al., 2016). In the uterus, the luminal AKR1B1 has been found to be involved in the polyol pathway during the conceptus peri-implantation period and, in porcine, its expression is downregulated as conceptus attaches to the endometrium (Steinhauser et al., 2016). On the other hand, uterine AKR1B1 is involved in prostaglandines synthesis in humans (Bresson et al., 2011) and pigs (Seo et al., 2014), thus contributing to prepare the endometrium for conceptus implantation as well as modulating the maternal immune system (Czyzyk et al., 2017). Our results, together with the fact that SP-AKR1B1 is linked with high *in vivo* pregnancy outcomes (Pérez-Patiño et al., 2018), suggest that SP-AKR1B1 could act jointly with the endometrial AKR1B1 to prepare the uterine environment for conceptus implantation. Another possibility could be that this protein has a direct impact on conceptus. In this sense, SP has been reported to exert a positive effect on pregnancy outcomes, specifically improving implantation and pregnancy rates in humans (Crawford et al., 2015; Saccone et al., 2019) and even modifying embryo gene expression in pigs (Martinez et al., 2020). Thus, it cannot be discarded the influence of SP-AKR1B1 on the improvement of embryo survival and even implantation, through the modulation of the uterine environment. In this regard, all these functions could be driven by both the soluble form of the protein in SP or by that contained in extracellular vesicles (EVs). SP EVs regulate sperm function through its integration to sperm membrane (Leahy et al., 2020) and their action on the female immune system (Zhang et al., 2020). Interestingly, bovine AKR1B1 has been reported to be associated to epididymal EVs (Frenette et al., 2006) and, in humans, seminal EVs have been found to contain aldose reductase (Zhang et al., 2020). In pigs, however, whether this protein is also in the cargo of SP EVs and participates in fertilization or embryo development remains to be explored.

AKR1B1 has been extensively demonstrated to play a role in female and male reproductive physiology. In pigs, SP-AKR1B1 has been reported to exert a positive impact on *in vivo* fertility outcomes. This study demonstrated that all male genital organs (except bulbourethral glands) are able to express AKR1B1. We also found that the concentration of AKR1B1 was higher in the post-SRF, suggesting that seminal vesicles could be the main contributor of this SP protein to the final ejaculate. Our results also indicated that SP-AKR1B1 is not associated to the quality and functionality parameters of sperm. These findings, together with the fact that this protein has been shown to be positively related to *in vivo* fertility (Pérez-Patiño et al., 2018), suggest that it could play an active role in the female reproductive tract, promoting sperm fecundity or even embryo development. Thus, further studies to determine the exact mechanism through which SP-AKR1B1 has a positive influence on *in vivo* fertility outcomes should be conducted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YM-O, MY, and IB: conceptualization. YM-O, EV-V, and ML: methodology. YM-O, JR-M, MY, and IB: formal analysis and investigation. YM-O and EV-V: writing—original draft preparation. JR-M, JR, MY, and IB: writing—review and editing. JR and MY: funding acquisition. IB and MY: supervision. All authors read and agreed to the published version of the manuscript.

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Paper III

Metabolomic fingerprinting of pig seminal plasma identifies *in vivo* fertility biomarkers.

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RESEARCH Open Access

Metabolomic fingerprinting of pig seminal plasma identifies *in vivo* fertility biomarkers

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Abstract

Background: Metabolomic approaches, which include the study of low molecular weight molecules, are an emerging -omics technology useful for identification of biomarkers. In this field, nuclear magnetic resonance (NMR) spectroscopy has already been used to uncover (in) fertility biomarkers in the seminal plasma (SP) of several mammalian species. However, NMR studies profiling the porcine SP metabolome to uncover *in vivo* fertility biomarkers are yet to be carried out. Thus, this study aimed to evaluate the putative relationship between SP-metabolites and *in vivo* fertility outcomes. To this end, 24 entire ejaculates (three ejaculates per boar) were collected from artificial insemination (AI)-boars throughout a year (one ejaculate every 4 months). Immediately after collection, ejaculates were centrifuged to obtain SP-samples, which were stored for subsequent metabolomic analysis by NMR spectroscopy. Fertility outcomes from 1525 inseminations were recorded over a year, including farrowing rate, litter size, stillbirths per litter and the duration of pregnancy.

Results: A total of 24 metabolites were identified and quantified in all SP-samples. Receiver operating characteristic (ROC) curve analysis showed that lactate levels in SP had discriminative capacity for farrowing rate (area under the curve [AUC] = 0.764) while carnitine (AUC = 0.847), hypotaurine (AUC = 0.819), sn-glycero-3-phosphocholine (AUC = 0.833), glutamate (AUC = 0.799) and glucose (AUC = 0.750) showed it for litter size. Similarly, citrate (AUC = 0.743), creatine (AUC = 0.812), phenylalanine (AUC = 0.750), tyrosine (AUC = 0.753) and malonate (AUC = 0.868) levels had discriminative capacity for stillbirths per litter; and malonate (AUC = 0.767) and fumarate (AUC = 0.868) levels for gestation length.

Conclusions: The assessment of selected SP-metabolites in ejaculates through NMR spectroscopy could be considered as a promising non-invasive tool to predict *in vivo* fertility outcomes in pigs. Moreover, supplementing AI-doses with specific metabolites should also be envisaged as a way to improve their fertility potential.

Keywords: Artificial insemination, *in vivo* fertility, Metabolomics, NMR, Pregnancy outcomes, Seminal plasma

Background

Predicting the reproductive potential of sires remains a pending challenge for the livestock industry. This is of particular relevance for the swine sector, whose breeding is mainly based on the use of artificial insemination (AI), an essential tool applied globally to improve reproductive efficiency [1]. Over the last few years, the enhancement of AI-procedures in this species has led to (1) a decrease in the sperm numbers per AI-dose, and (2) a reduction in the number of AI performed per sow, without modifying *in vivo* fertility outcomes [2]. This situation entails that a higher number of AI-doses are elaborated from a single AI-boar and a higher number of sows are inseminated with a single AI-boar, which

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leads to an increase in the reproductive and economic repercussion of AI-boars on swine farms [2]. Although AI-boars are selected on the basis of their genetic merit and the results obtained by routine sperm analyses (which include sperm concentration, morphology and motility), differences among AI-boars on *in vivo* fertility outcomes are still notable [3, 4]. For this reason, many efforts have been made to uncover biomarkers capable to predict *in vivo* fertility outcomes.

During the past few years, special emphasis has been paid to identify these biomarkers in seminal plasma (SP), a heterogenous fluid secreted by the epididymis and accessory sex glands [5]. This fluid has been poised as a potential source of biomarkers, due to its complex composition and its ability to interact with sperm and the female genital tract, playing a key role in sperm physiology and maternal environment modulation [6–8]. In this sense, high-throughput technologies (including genomics, lipidomics, proteomics, metabolomics and transcriptomics) may hold the key for uncovering reliable fertility biomarkers in SP, since they provide a more in-depth understanding of reproductive processes [9]. In the last decade, many studies conducted in mammalian SP have employed these novel technologies to collect large amounts of data to discover novel fertility biomarkers [10–13].

Metabolomics is the last emerging -omics technology that has become a promising tool to identify biomarkers of (in) fertility [11, 14]. This high-throughput method allows for the study of cells, tissues and biological fluids by evaluating metabolic products, which are the finished outputs of cellular processes [11, 15]. The identification of (in) fertility biomarkers in SP through metabolomics approaches has been extensively reported in several mammalian species, including human [16–19], porcine [20] and bovine [21, 22]. In pigs, Zhang et al. (2021) compared the SP metabolome obtained by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry between boars with high and low conception rates after AI (< 70 sows inseminated per boar), identifying some SP-metabolites (such as Pro-Asn, Ile-Tyr, and D-Biotin) as potential fertility biomarkers [20]. However, neither the concentration of SP-metabolites, nor the putative relationship between SP-metabolites and other *in vivo* fertility outcomes (e.g. litter size, stillbirths per litter or gestation duration) was reported by these authors.

The aim of this study was to evaluate the relationship between the presence/concentration of SP-metabolites and reproductive performance (including farrowing rate, litter size, stillbirths per litter and duration of pregnancy) of liquid-stored pig semen using Nuclear Magnetic Resonance (NMR) spectroscopy. To achieve this goal, a total of eight AI-boars were included in the study and data from 1,525 inseminations were recorded over a year (> 100 sows inseminated per boar). Using this approach, the present study was able to identify several SP-metabolites able to potentially predict AI outcomes.

Methods

Experimental design

A total of 24 entire ejaculates were collected from eight AI-boars (three ejaculates per boar) throughout a year (one ejaculate every 4 months). Immediately after collection, ejaculates were centrifuged to obtain SP-samples, which were stored (– 80 °C) for subsequent metabolomic analysis. Seminal AI-doses ($2{,}400 \times 10^6$ spermatozoa in 80 mL) were prepared from these AI-boars and used to inseminate (cervically; two times per estrus) a total of 1,525 weaned multiparous sows (1–7 litters produced) throughout a year. These sows (Landrace and Large White) were housed in different farms in Spain with comparable management conditions. Each boar serviced more than 100 sows.

Fertility outcomes were recorded from each AI-boar included in the study during the same year that SP-samples were collected and AI were performed. Recorded fertility variables were: (1) farrowing rate (percentage of inseminated sows that farrowed), (2) litter size (total number of piglets born per litter), (3) number of stillbirths per litter, and (4) duration of pregnancy (days). These fertility records were corrected for farm-related parameters and sows using the multivariate statistical model described by Broekhuijse et al. [23]. This model allows isolating the direct boar effect on each *in vivo* fertility parameter.

Boars and ejaculates

All ejaculates were collected from AI-boars housed in a Spanish AI-Center (AIM Iberica, Topigs Norsvin Spain SLU, Calasparra, Murcia, Spain). This center fulfilled the Spanish (ES300130640127, August 2006) and European (ES13RS04P, July 2012) rules in matters of animal health, collection of boar ejaculates and commercialization of AI-doses. As no animal was manipulated by the authors but rather the AI-Center provided AI-doses and fertility data, no permission from an Ethics Committee was required.

The entire ejaculates used in this study were collected from healthy, mature (12 to 36 months), fertile boars from different breeds (Landrace and Large White) using a semi-automatic collection system (Collectis®, IMV Technologies, L'Aigle, France). These boars were included in an AI-program and subjected to regular ejaculate collection (twice per week) for producing seminal AI-doses. The entire ejaculates included in this study satisfied the semen quality limits required to produce commercial AI-doses (sperm concentration > 200×10^6

sperm/mL; sperm motility > 70%; sperm with normal morphology > 75%).

Boars were housed in individual pens with controlled temperature (15–25 °C) and light (16 h; natural and artificial). Animals had free access to water and were fed with agricultural feedstuff in agreement with the nutritional requirements of AI-boars.

Seminal plasma processing and storage

For SP-harvesting, the entire ejaculates were centrifuged (1,500 × *g* for 10 min at room temperature [Rotofix 32A; Hettich Centrifuge UK, Newport Pagnell, Buckinghamshire, England, UK]) twice immediately after ejaculate collection. The resulting second supernatants (SP-samples) were subsequently analyzed (Eclipse E400; Nikon, Tokyo, Japan) to warrant the absence of sperm. Finally, SP-samples were stored in 2-mL cryotubes at − 80 °C (Ultra Low Freezer; Haier Inc., Qingdao, China) until metabolomic profiling was carried out.

^{1}H NMR analysis

The SP-samples were thawed on ice and one of the aliquots (500 μL) used. Each aliquot was vortexed and centrifuged through 0.5 mL Amicon® Ultra Centrifugal Filters (14,000 × *g* at 4 °C for 90 min) for discarding proteins and cell debris. Then, 100 μL of PBS containing 10% D_2O with 0.33% of DSS (Merck KgaA, Darmstadt, Germany; pH 7.4) were added to the eluted fractions and transferred into a 5-mm Wilmad® NMR tube (Merck KgaA), where 100 μL of D_2O was added. Finally, the ^{1}H NMR profile was acquired.

^{1}H NMR spectra

A Bruker 600-MHz AVANCE III NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a ^{1}H frequency of 600.13 MHz and 300 K with a previous equilibration time (10 min) was used to obtain NMR spectra. The 1D-^{1}H-nuclear Overhauser effect spectroscopy (1D-NOESY) pulse sequence from the Bruker library was used. The parameters applied were: (1) mixing time: 100 ms (d8); (2) recovery delay: 2 s (d1); (3) 90° pulse: 10.39 μs (p1); (4) spectral width: 7211.539 Hz; (5) spectral size: 32 k; (6) number of scans: 128; and (7) acquisition time: 2.27 s.

Data processing and analysis

The Chenomx 8.0 profiler software was used for processing and analyzing spectra. This software delivers tools for automatic phase, baseline correction, reference calibration and libraries of metabolites for profiling. The concentration of each metabolite identified in SP was calculated based on DSS concentration (0.216 mmol/L).

Statistical analysis

All analyses were carried out using R software (version 4.0.2; https://www.r-project.org/). For all analysis, the level of significance was set at $P \leq 0.05$. Statistical analysis of NMR data was performed in two steps: a) numeric (fertility) vs. numeric (SP-metabolite concentration) variables, and b) categoric (fertility parameter) vs. numeric (SP-metabolite concentration) variables.

First, numerical analysis, namely Pearson correlations, were used to preliminary evaluate the potential linear relationship between SP-metabolite concentration and each fertility parameter.

Onwards, data were split into two different groups for each reproductive parameter (farrowing rates, litter size, stillbirths per litter and pregnancy length). Samples with values lower than the median were classified as negative farrowing rate, decreased litter size and stillbirths per litter, and shorter pregnancy duration; samples with values higher than the median were classified as positive farrowing rate, increased litter size and stillbirths per litter, and longer pregnancy duration. This process was executed for each individual fertility parameter, yielding a specific categorization for each one.

To evaluate potential differences in SP-metabolite concentrations between fertility groups, a Wilcoxon rank sum test (equivalent to Mann-Whitney U test) was performed. As opposed to *t*-test, Wilcoxon does not assume normal distribution of samples, which did not occur in some cases. A multivariate analysis was also carried out to evaluate putative inter-metabolite relationships and patterns that could predict fertility outcomes. In this sense, a Bayesian logistic regression was used, treating the groups above the median as success (1) and those below the median as failure (0). An individual model was run for each of the fertility parameters considering all the metabolites as potential predictors, using the R package 'rstanarm' (R package version 2.21.1; [24]), with non-informative prior distributions, high resolution sampling of the posterior distribution (adapt_delta = 0.99) and 4,000 iterations. The remaining parameters of the models were left by default. The Bayesian framework was selected over the classical frequentist one because of the structure of data. Usually, with a higher number of features (or predictors) than samples, as in the present dataset, models tend to get overfitted. While classical regression models rely on confidence intervals to estimate their reliability, the Bayesian framework estimates the whole posterior (the approximately 'real') distribution and allows quantifying the uncertainty of coefficients and predictions accordingly. This methodology is particularly of interest not only for having a good sense of how accurate predictions are, but also for improving the models as more information about data becomes

available (e.g., knowledge about the mean or the range of the 'true' distribution of the predictors).

As a last step to assess the predictability of the different fertility parameters, two additional analyses were performed. A sparse partial least square discriminant analysis (sPLS-DA) model was run separately for each of the parameters, using the 'mixOmics' R package [25]. Similar to principal components analysis (PCA), this method is useful for identifying key features in the dataset. However, while PCA relies on maximizing the variance of the features in the principal components, sPLS-DA maximizes their covariance. Metabolites that were relevant in the sPLS-DA analysis and/or exhibited differences between groups were further tested in a Receiver Operating Characteristic (ROC) curve, using the 'pROC' package for R [26]. This method allows for further validation of the predictive performance of metabolites and provides a 'cut-off' or threshold value to discriminate (or predict) sample fertility ('high' or 'low'). Results are expressed as the area under the curve (AUC). The discriminant relevance was measured by the following AUC ranges: 0.0–0.5 = no discriminant value, 0.5–0.6 fail discriminant value, 0.6–0.7 poor discriminant value, 0.7–0.8 fair discriminant value, 0.8–0.9 good discriminant value, and 0.9–1 excellent discriminant value.

Results

Metabolite profile of pig SP

The ^{1}H-NMR profile allowed the identification and quantification of a total of 24 metabolites in pig SP-samples (see Supplementary Fig. 1). The identified metabolites were categorized in: i) amino acids ($n = 7$; alanine, glutamate, isoleucine, leucine, phenylalanine, tyrosine and valine); ii) alcohols ($n = 2$; ethanol and methanol); iii) saccharides ($n = 1$; glucose); iv) salts ($n = 7$; acetate, benzoate, citrate, formate, fumarate, lactate and malonate); and v) other organic compounds ($n = 5$; carnitine, creatine, creatine-phosphate, hypotaurine, myo-inositol, sn-glycero-3-phosphocholine and trimethylamine N-oxide).

Association between SP-metabolites and AI outcomes

Correlations between the concentration of SP-metabolites and *in vivo* fertility parameters were calculated (Fig. 1). Five SP-metabolites were found to be correlated ($P < 0.05$) with farrowing rate: lactate (R = – 0.62), leucine (R = 0.55), phenylalanine (R = 0.45), tyrosine (R = 0.49) and valine (R = 0.53). Moreover, two SP-metabolites were positively correlated ($P < 0.05$) with litter size: carnitine (R = 0.42) and hypotaurine (R = 0.51). Additionally, the number of stillbirths per litter was negatively correlated ($P < 0.05$) with nine SP-metabolites: citrate (R = – 0.42), creatine (R = – 0.51), creatine phosphate (R = – 0.46), isoleucine (R = – 0.47), leucine (R = – 0.46), methanol (R = – 0.53), phenylalanine (R = – 0.54), tyrosine (R = – 0.52) and valine (R = 0.57). Finally, the duration of gestation was negatively correlated ($P < 0.05$) with four SP-metabolites: citrate (R = – 0.51), creatine (R = – 0.45), methanol (R = – 0.63) and myo-inositol (R = – 0.59).

Bayesian multiple logistic regression analyses were carried out with the aim (i) to develop a potential predictive

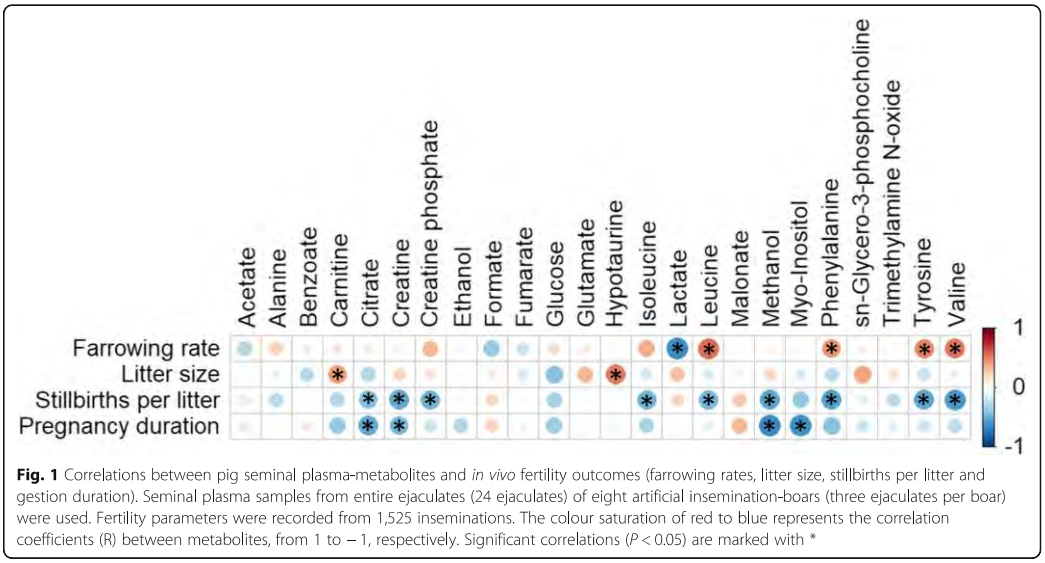

Fig. 1 Correlations between pig seminal plasma-metabolites and *in vivo* fertility outcomes (farrowing rates, litter size, stillbirths per litter and gestion duration). Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1,525 inseminations. The colour saturation of red to blue represents the correlation coefficients (R) between metabolites, from 1 to – 1, respectively. Significant correlations ($P < 0.05$) are marked with *

model, (ii) to quantify the relative contribution of each SP-metabolite to each *in vivo* fertility parameter, and (iii) to identify specific metabolite patterns that may have an influence on *in vivo* fertility parameters. However, no model showed a clear relationship with any of the reproductive outcomes (Supplementary Fig. 2A-D).

Relationship between SP-metabolites and farrowing rate

Boars were classified into two groups based on their farrowing rate deviation from the median: negative farrowing rate deviation (ranging from − 2.80 to − 1.60; $n = 4$) and positive farrowing rate deviation (ranging from 2.82 to 7.54; $n = 4$). Only the concentration of lactate in SP differed ($P < 0.05$) between groups, showing higher levels in SP-samples from boars with negative farrowing rate deviation (median ± SD; 1.90 mmol/L ± 0.508) compared to those with positive farrowing rate deviation (median ± SD; 1.22 mmol/L ± 0.585).

The sPLS-DA analysis was carried out to select the most predictive or discriminant features in the dataset to classify samples [27]. The sPLS-DA analysis for farrowing rates deviation using the first two components explained 49.9% of the total variance of the sample (Fig. 2A). The resulting plot showed two different groups: SP-samples from boars exhibiting negative farrowing rate deviation (blue) were mainly discriminated by the second component, whereas SP-samples from boars classified as positive farrowing rate deviation (red) were separated by the first component. The loadings plot, which shows the most relevant variable for a given component, revealed that whereas lactate and formate were the most important variables for the first component, trimethylamine N-oxide and alanine were the most relevant for the second one (Fig. 2B). ROC curve analysis indicated that only lactate was able to predict farrowing rate deviation ($P < 0.05$; Fig. 2C). Specifically, lactate showed a fair discriminant value with an AUC of 0.764.

Relationship between SP-metabolites and litter size

Boars were classified into two groups depending on their litter size deviation from the median: reduced litter size (ranging from − 0.40 to 0.02; $n = 4$) and increased litter size (ranging from 0.11 to 0.52; $n = 4$) deviation. Concentrations of carnitine, hypotaurine, sn-glycero-3-phosphocholine and glutamate in SP differed ($P < 0.05$) between groups, displaying higher levels in SP-samples from boars with increased litter size deviation than in SP-samples from boars with reduced litter size deviation (median ± SD; for carnitine: 0.82 mmol/L ± 0.223 vs. 0.43 mmol/L ± 0.244; for glutamate: 1.71 mmol/L ± 0.437 vs. 1.33 mmol/L ± 0.607; for hypotaurine: 2.85 mmol/L ± 0.604 vs. 1.77 mmol/L ± 0.813; for sn-glycero-3-phosphocholine: 6.45 mmol/L ± 1.373 vs. 4.69 mmol/L ± 1.932, respectively). Concentration of glucose in SP also differed ($P < 0.05$) between groups, showing the opposite pattern to the aforementioned metabolites; indeed, SP-samples from boars with increased litter size deviation (median ± SD; 0.24 mmol/L ± 0.273) exhibited lower glucose concentration than those with decreased litter size deviation (median ± SD; 0.79 mmol/L ± 0.245).

The sPLS-DA analysis (Fig. 3A) showed that the first two components explained 53.9% of the total variance of the sample. The plot showed two different groups; SP-samples from boars classified with increased litter size deviation (red) were mainly influenced by the second component, whereas SP-samples from boars classified with decreased litter size deviation (blue) were mainly affected by the first component. The loadings plot revealed that while carnitine, hypotaurine, sn-glycero-3-phosphocholine and glucose strongly influenced the first component, glutamate and methanol had that effect on the second component (Fig. 3B). ROC curve analysis showed that all SP-metabolites identified as relevant by the loadings plot had a significant AUC ($P < 0.05$; Fig. 3C). Specifically, carnitine showed the highest AUC of 0.840, hypotaurine displayed an AUC of 0.819, sn-glycero-3-phosphocholine showed an AUC of 0.833, glucose exhibited an AUC of 0.750, and glutamate had an AUC of 0.799. Thus, the ROC curve showed that while carnitine, hypotaurine, sn-glycero-3-phosphocholine and glutamate exhibited a good discriminant value for predicting litter size due to their high AUC (ranging 0.8–0.9), glucose had a fair discriminant predictive value (ranging 0.6–0.7) for litter size.

Relationship between SP-metabolites and the number of stillbirths per litter

Boars were categorized into two groups depending on their stillbirths per litter deviation from the median: decreased stillbirths per litter deviation (ranging from − 0.10 to 0.02; $n = 4$) and increased stillbirths per litter deviation (ranging from 0.05 to 0.14; $n = 4$). Concentrations of citrate, creatine, phenylalanine and tyrosine in SP differed ($P < 0.05$) between groups: the SP-samples from boars with decreased stillbirths per litter deviation showing higher concentrations than those from boars with increased stillbirths per litter (median ± SD; for citrate: 7.10 mmol/L ± 1.738 vs. 4.95 mmol/L ± 2.192; for creatine: 0.54 mmol/L ± 0.158 vs. 0.31 mmol/L ± 0.156; for phenylalanine: 0.03 mmol/L ± 0.011 vs. 0.02 mmol/L ± 0.012; for tyrosine: 0.03 mmol/L ± 0.017 vs. 0.02 mmol/L ± 0.016, respectively). In an opposite manner, malonate was found to be higher ($P < 0.05$) in SP-samples from boars with increased stillbirths per litter (median ± SD; 0.16 mmol/L ± 0.064) than in those from boars with decreased stillbirths per litter (median ± SD; 0.09 mmol/L ± 0.054).

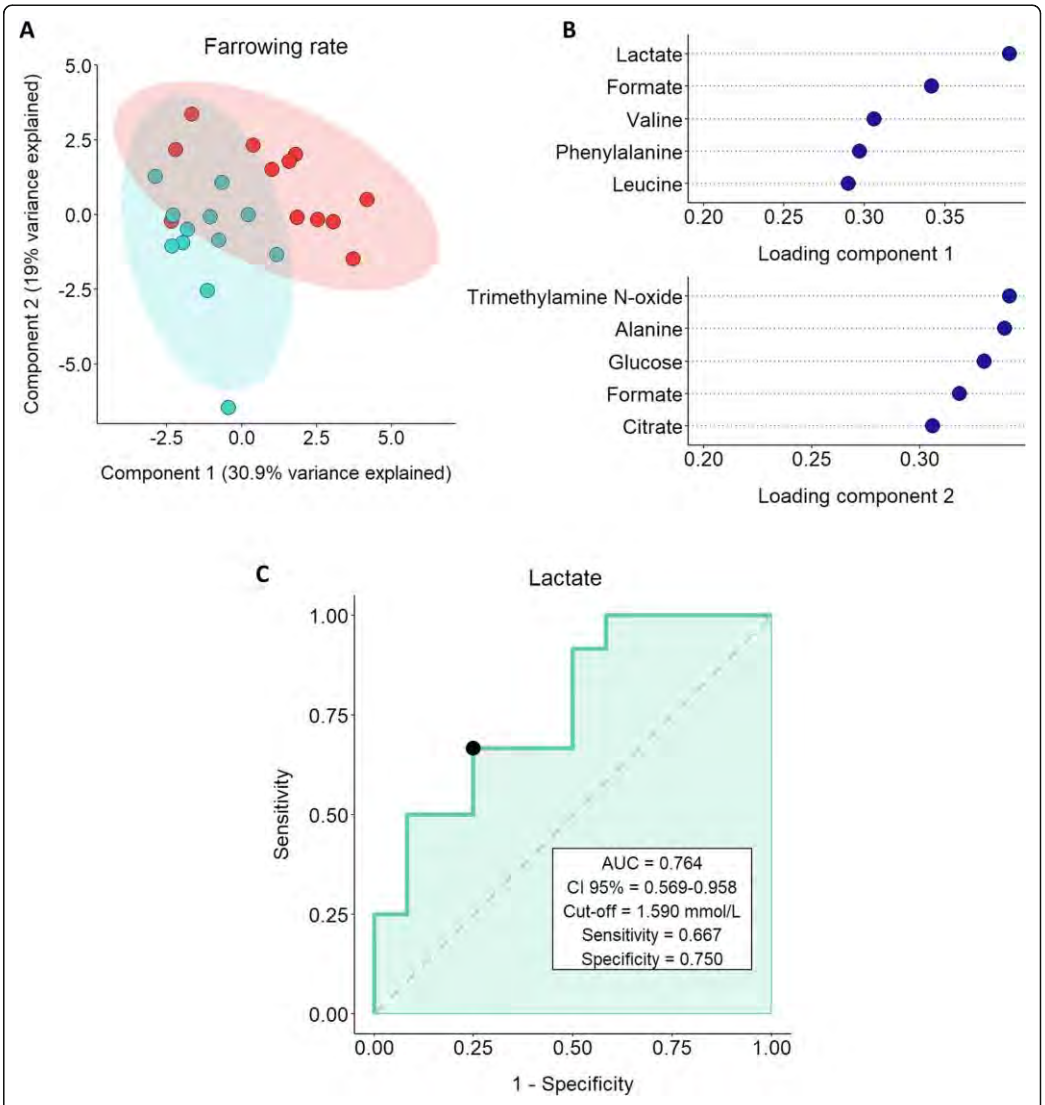

Fig. 2 **A** sPLS-DA analysis for farrowing rates deviation showing sample distribution in component 1 and component 2. Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1525 inseminations. The colored areas represent the 95% confidence interval. Boars classified with positive farrowing rate deviation (ranging from 2.82 to 7.54; $n = 4$) are represented in red and those with negative farrowing rate deviation (ranging from − 2.80 to − 1.60; $n = 4$) are shown in blue. Each dot symbolizes an ejaculate. **B** Loading plot for components 1 and 2 for the sPLS-DA model. Variables are ranked by the absolute values of their loadings. **C** Receiver operating characteristic (ROC) curve analysis for lactate concentration in seminal plasma and farrowing rate deviation. Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1,525 inseminations. The plot shows the ability of a given metabolite to discriminate farrowing rate of semen doses. AUC: area under the curve; CI: confidence interval

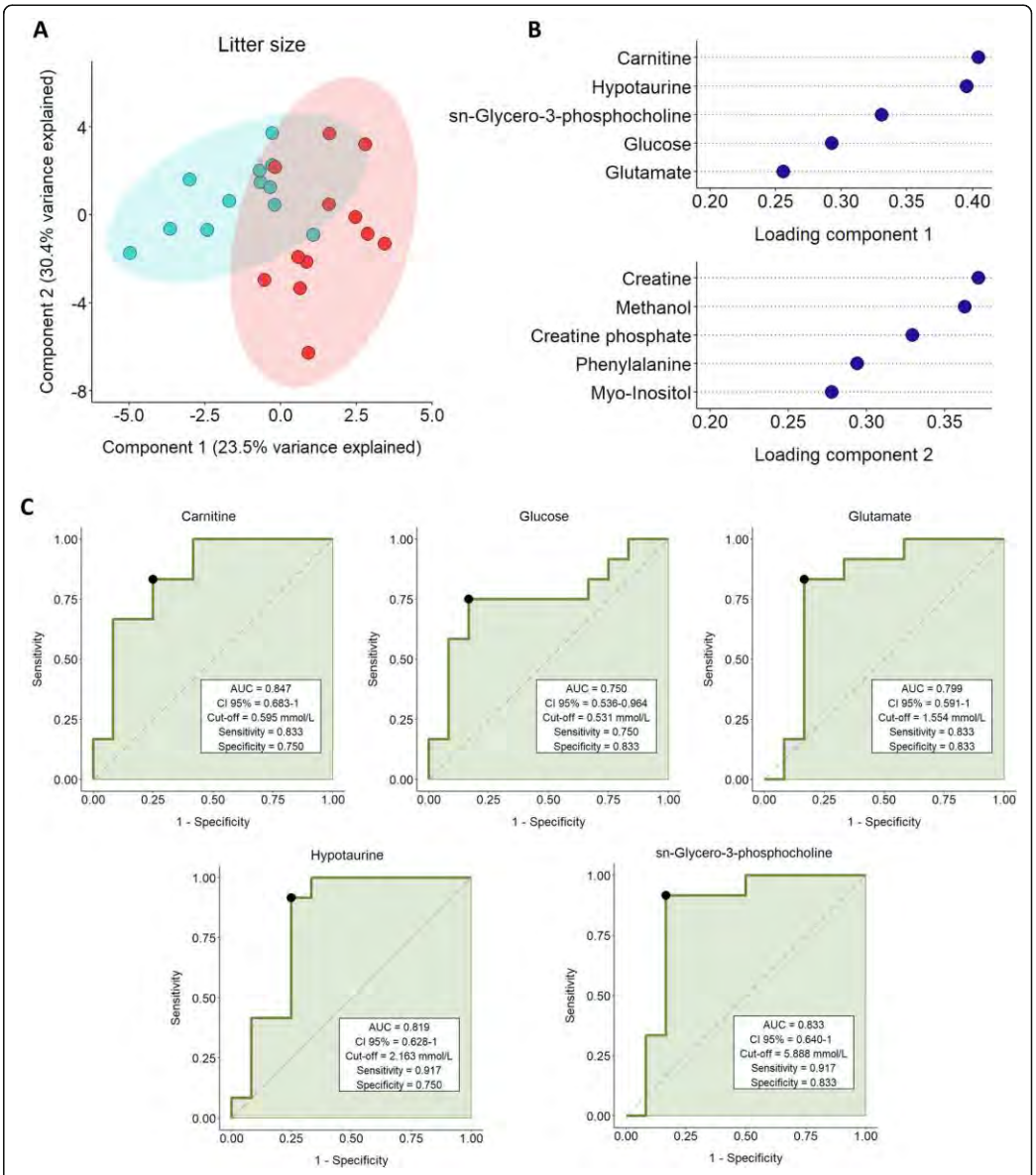

Fig. 3 A sPLS-DA analysis for litter size deviation showing sample distribution in component 1 and component 2**.** Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1525 inseminations. The colored areas represent the 95% confidence interval. Boars with decreased litter size deviation (ranging from − 0.40 to 0.02; $n = 4$) are represented in blue and those with increased litter size deviation (ranging from 0.11 to 0.52; $n = 4$) are shown in red. Each dot symbolizes an ejaculate. **B** Loading plot for components 1 and 2 for the sPLS-DA model. Variables are ranked by the absolute values of their loadings. **C** Receiver operating characteristic (ROC) curve analysis for carnitine, glucose, sn-glycero-3-phosphocholine, glutamate and hypotaurine concentrations in seminal plasma and litter size deviation. Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1,525 inseminations. The plot shows the ability of the metabolites to discriminate litter size of semen doses. AUC: area under the curve; CI: confidence interval

Regarding the sPLS-DA analysis (Fig. 4A), the first two components were found to explain 51.8% of the total variance. Moreover, two different groups were observed: while SP-samples from boars with decreased deviation in the number of stillbirths per litter (blue) were mainly influenced by both components, SP-samples from boars with increased deviation (red) were influenced by the second component. The loadings plot revealed that the first component was mainly influenced by creatine and malonate, and the second component by malonate, benzoate and formate (Fig. 4B). ROC curve analysis showed that the AUC was significant ($P < 0.05$; Fig. 4C) for citrate, creatine, malonate, phenylalanine and tyrosine. Specifically, citrate exhibited an AUC of 0.743, creatine displayed an AUC of 0.812, malonate showed an AUC of 0.868, phenylalanine displayed an AUC of 0.750, and tyrosine showed an AUC of 0.753. Therefore, the ROC curve revealed that while creatine and malonate had a good discriminant value for predicting stillbirths per litter (as their AUC ranged from 0.8 to 0.9), citrate, creatine and tyrosine displayed a fair discriminant strength (as their AUC ranged from 0.7 to 0.8).

Relationship between SP-metabolites and duration of gestation

Boars were classified into two groups depending on the deviation of gestation duration from the median, i.e. shorter gestation duration (ranging from − 0.85 to 0.03; $n = 4$) and longer gestation duration (ranging from 0.10 to 0.52; $n = 4$) deviation. Concentration of malonate in SP differed ($P < 0.05$) between groups, showing higher levels in SP-samples from boars with longer gestation duration deviation (median ± SD; 0.16 mmol/L ± 0.072) compared to those from boars with shorter gestation duration deviation (median ± SD; 0.09 mmol/L ± 0.030). On the contrary, fumarate exhibited higher levels ($P < 0.05$) in SP-samples from boars with shorter gestation duration (median ± SD; 0.01 mmol/L ± 0.002) than in those from boars with longer gestation duration (median ± SD; 0.004 mmol/L ± 0.002).

sPLS-DA analysis for gestation duration showed that the first two components explained 33.6% of the total variance (Fig. 5A). Again, two different groups were identified: while SP-samples from boars classified with a shorter deviation in the gestation duration (blue) were mainly affected by the second component, SP-samples from boars with longer deviation (red) were equally influenced by both components. The loadings plot revealed that the first component was strongly influenced by malonate, and the second component by glutamate, sn-glycero-3-phosphocholine and carnitine (Fig. 5B). For these SP-metabolites, both malonate and fumarate showed a significant ROC curve ($P < 0.05$; Fig. 5C). Specifically, malonate exhibited an AUC of 0.868 and fumarate showed an AUC of 0.767. Considering these results, the ROC curve revealed that while malonate had a good discriminant value for predicting gestation duration, fumarate showed a fair discriminant predictive value for this parameter.

Discussion

In the last years, metabolite identification and quantification for male infertility assessment has become an emerging area of research [9, 10, 28]. In this field, NMR spectroscopy is one of the three most common analytical methods for metabolite profiling [28]. The present report evaluated the potential relationship between SP-metabolite concentrations and AI outcomes in pigs using NMR approaches, figuring out which SP-metabolites could be used as *in vivo* fertility biomarkers. Specifically, this study demonstrated that: i) the concentration of SP-lactate was related to farrowing rate; ii) concentrations of carnitine, hypotaurine, sn-glycero-3-phosphocholine glutamate and glucose in SP were associated with litter size; iii) concentrations of citrate, creatine, malonate, phenylalanine and tyrosine in SP were related to the number of stillbirths per litter; and iv) concentrations of malonate and fumarate in SP were associated to gestation duration.

In accordance with our previous report [29], this study identified and quantified a total of 24 SP-metabolites. In addition, the results of the present work showed that several SP-metabolites were related to specific *in vivo* fertility parameters. However, since all the relationships were found to be moderate (as Pearson correlation coefficients were lower than 0.6) and no SP-metabolite pattern for specific fertility parameters was observed using Bayesian multiple logistic models, sPLS-DA and ROC analysis were run. Using these statistic tools, potential biomarkers for all the assessed reproductive performance variables were identified.

The results of the present study evidenced that lactate was the only SP-metabolite related to farrowing rate. These results differ from those reported by Zhang et al. who found that several amino acids and D-biotin in SP were related with conception rates in pigs [20]. Differences in (1) the analytical method (mass spectrometry vs. NMR) and in (2) the analysis of fertility records, since the work of Zhang et al. did not take other parameters that could influence conception rates (sows, farms …) into consideration [20], may contribute to explain the divergent results between both studies. In the present work, the highest lactate concentrations were found in SP-samples from boars classified with negative farrowing rate deviation. It is well known that lactate is one of the main non-monosaccharide substrates for sperm in bulls [30], stallions [31], men [32] and boars [32, 33]. Non-oxidative metabolism of pig sperm has been shown to

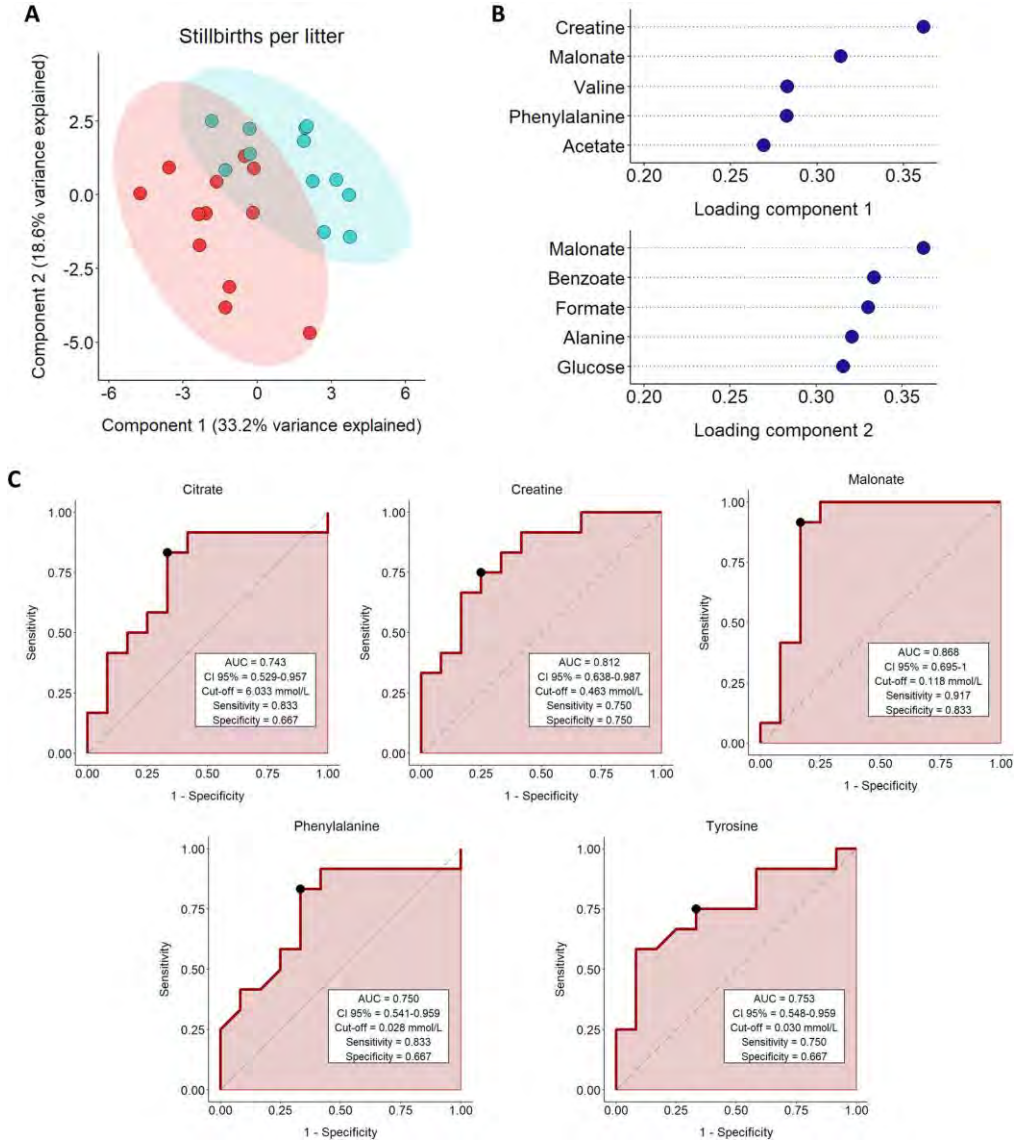

Fig. 4 **A** sPLS-DA analysis for the number stillbirths per litter deviation showing sample distribution in component 1 and component 2. Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1,525 inseminations. The colored areas represent the 95% confidence interval. Boars with decreased stillbirths per litter deviation (ranging from − 0.10 to 0.02; $n = 4$) are represented in blue and those with increased stillbirths per litter deviation (ranging from 0.05 to 0.14; $n = 4$) are shown in red. Each dot symbolizes an ejaculate. **B** Loading plot for components 1 and 2 for the sPLS-DA model. Variables are ranked by the absolute values of their loadings. **C** Receiver operating characteristic (ROC) curve analysis for citrate, creatine, phenylalanine, tyrosine and malonate and stillbirths per litter. They show the ability of metabolites to discriminate the number of stillbirths per litter after artificial insemination with semen doses. AUC: area under the curve; CI: confidence interval

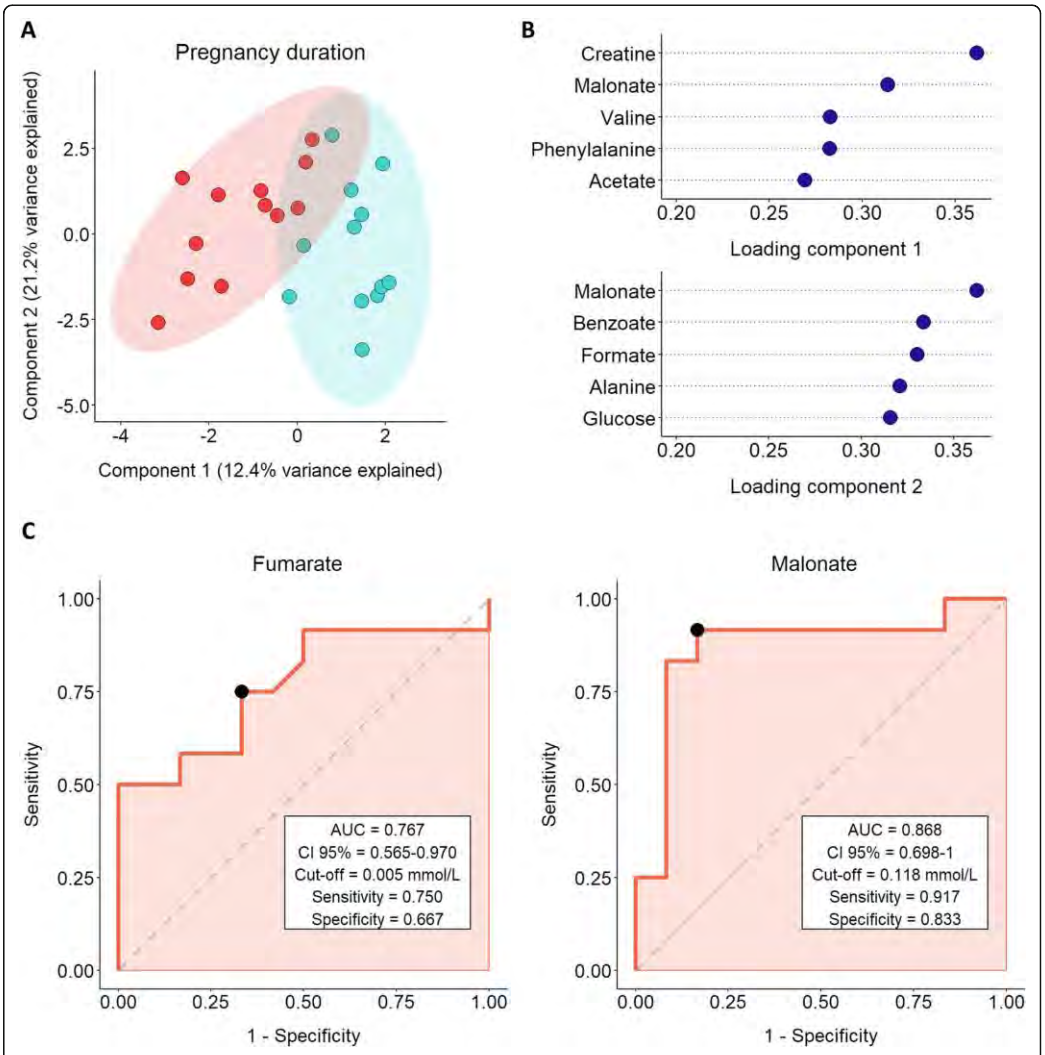

Fig. 5 **A** sPLS-DA analysis for gestation duration deviation showing sample distribution in component 1 and component 2. Seminal plasma samples from entire ejaculates (24 ejaculates) of eight artificial insemination-boars (three ejaculates per boar) were used. Fertility parameters were recorded from 1,525 inseminations. The colored areas represent the 95% confidence interval. Boars with longer gestation duration deviation (ranging from 0.10 to 0.52; $n = 4$) are represented in red and those with shorter gestation duration deviation (ranging from − 0.85 to 0.03; $n = 4$) are shown in blue. Each dot symbolizes an ejaculate. **B** Loading plot for components 1 and 2 for the sPLS-DA model. Variables are ranked by the absolute values of their loadings. **C** Receiver operating characteristic (ROC) curve analysis for malonate and fumarate and pregnancy duration. They show the ability of metabolites to discriminate the number of stillbirths per litter after artificial insemination with semen doses. AUC: area under the curve; CI: confidence interval

consume lactate [34], which is transformed into pyruvate through lactate dehydrogenase to produce ATP [35]. Considering these findings, one could assume that sperm from boars classified with positive farrowing rate deviation could better metabolize lactate for energy production, thereby leading to lower SP-lactate concentration, as confirmed by the present study. However, it is worth mentioning that these results are not in agreement with previous findings reported in cattle, in which the highest lactate levels were found in the SP of high

fertility bulls [30], and in men, as infertile patients had lower SP-lactate levels than fertile controls [16, 18, 36, 37]. These differences could be attributed to: (1) different metabolic sperm strategies, as while pig sperm can use lactate as an energy source, the rate between oxidative phosphorylation and glycolysis is higher in bovine sperm [38]; and/or (2) differences in SP composition as a result of differences in mating strategies between species [39, 40].

Regarding the litter size, the present study showed that concentrations of glucose, carnitine, hypotaurine, sn-glycero-3-phosphocholine and glutamate were related with this fertility parameter. Interestingly, the ROC curve revealed that these SP-metabolites had a discriminating ability to predict the litter size, so that all the four could be considered as promising biomarkers for this AI outcome.

It is well known that glucose is one of the main monosaccharides used by mammalian sperm to produce energy [32, 41]. The present study reported that boars classified with an increased litter size deviation exhibited lower glucose concentrations in their SP than those with a decreased litter size deviation. Similar findings were reported in humans, in which men with idiopathic infertility had higher glucose levels in their SP compared to healthy individuals [17]. The most feasible explanation for such findings would be that sperm from boars with a decreased litter size would consume less glucose from SP; thus, glucose would be extracellularly accumulated. This hypothesis would be in agreement with the existing literature, as the supplementation of semen extenders with glucose has been reported to increase sperm motility and ATP concentration in humans [42]. Taken these data together, it could be suggested that low levels of glucose in SP are beneficial for both sperm physiology and reproductive performance.

Carnitine is an antioxidant that has been widely demonstrated to be involved in mammalian sperm motility [43, 44]. Moreover, a protective role of this antioxidant on DNA and plasma membrane oxidation damage in humans [44, 45] and pigs [46] has also been reported. In addition, dietary carnitine supplementation in boars has been proved to improve sperm quality parameters [47, 48]. In agreement with these studies, the results reported herein indicate that boars with an increased litter size deviation exhibit the highest SP-concentration of this metabolite. These results are in accordance with the study of Zöpfgen et al., who found that infertile men had lower SP-levels of carnitine than their fertile counterparts [49]. Nevertheless, this result, together with the aforementioned findings, open the possibility of using the measurement of carnitine in SP as a potential litter size biomarker.

Hypotaurine is an antioxidant present in human SP and sperm [50, 51]. The present study found a positive influence of SP-hypotaurine on litter size. This relationship could be driven by both an effect on sperm and/or oocyte fertilization. With regard to sperm, the addition of hypotaurine to cryopreservation media has been reported to exert a positive effect on sperm quality and functionality parameters in sheep [52] and humans [51, 53]. In addition, sperm from bulls with high fertility records also have high hypotaurine levels [54]. While, considering all this evidence, one could surmise that SP-hypotaurine has a positive impact on pig sperm physiology, further studies are required to confirm this hypothesis. On the other hand, supplementation of *in vitro* culture media with hypotaurine increases embryo cleavage and, in consequence, embryo development in bovine [55] and improves the intracellular oxidative status of pre-implantational porcine embryos [56]. Thus, SP-hypotaurine could also affect early embryo development stages, thus increasing litter size.

Glutamate is an amino acid involved in cellular energy production and in the synthesis of many other amino acids and nucleotides [18]. Low levels of SP-glutamate have been related to several forms of infertility in humans [18, 37]. In agreement with these results, the present study found that higher levels of SP-glutamate were related to increased litter size deviation. Based on these data, the effect of glutamate on AI outcomes could be driven by its repercussion on sperm, as equine intracellular glutamate has been proposed: i) to contribute to sperm functionality through its metabolization via non-canonical pathways; and ii) to be exchanged for extracellular cysteine to produce reduced glutathione [57]. Nonetheless, before could glutamate be used as a litter size biomarker, the aforementioned hypothesis should be tested in the pig.

Finally, sn-glycero-3-phosphocholine, which is involved in glycerophospholipid metabolism, has been reported to play a vital role in sperm capacitation and acrosome reaction in rats [58]. The results of the present study showed a positive relationship between sn-glycero-3-phosphocholine concentration in SP and high litter size. These results seem to agree with previous studies performed in other species, in which infertile men were observed to exhibit lower sn-glycero-3-phosphocholine levels in their SP compared to their fertile counterparts [59]. In addition, it has been reported that rat sperm head accumulates lipid metabolites as a result of sn-glycero-3-phosphocholine metabolism during acrosome reaction, which could have an involvement in sperm-oocyte interaction and even in gamete fusion [58]. Considering all these findings, further research addressing the specific role played by SP-sn-glycero-3-phosphocholine in pig fertility is warranted.

The present study also evaluated the relationship between SP-metabolites and stillbirths per litter. Citrate, creatine, phenylalanine and tyrosine were observed to be promising biomarkers for stillbirths per litter due to their ROC curves. In this sense, citrate is involved in the Krebs cycle, which is the most relevant metabolic pathway for energy production [60]. The results of the present study revealed that high SP concentrations of this metabolite were related to a low number of stillbirths per litter. These results came as a surprise considering that low levels of SP-citrate have been observed in high-fertility bulls [21] and SP-citrate has been widely proposed as a biomarker for different human infertility forms [18, 36, 61]. Considering the opposite trend of the results presented herein, the exact mechanism through which SP-citrate could positively influence AI outcomes needs to be clarified in future studies.

Creatine is involved in the regulation of ATP and both the supplementation of *in vitro* fertilization medium with creatine [62] and the presence of this metabolite in SP [63] have been reported to influence sperm physiology in terms of motility and viability in humans [62, 63]. Interestingly, the present study found that high SP-creatine concentration was associated with decreased stillbirths per litter deviation. This result may be explained by the fact that creatine has been found to enhance fertilization and promote blastocyst and normal embryo development [62]. Consequently, although this should be further confirmed, it could be posited that high SP-creatine has a positive effect on both gametes, thus improving AI outcomes and decreasing the number of stillbirths per litter.

Phenylalanine and tyrosine, amino acids involved in the same metabolic pathway [64], were found to be higher in SP-samples from boars with decreased stillbirths per litter deviation. While, to the best of our knowledge, no information about the effect of tyrosine on sperm physiology has been reported, phenylalanine is known to stimulate the ability of human sperm to capacitate and undergo acrosomal exocytosis [65]. In cattle, phenylalanine levels in SP are positively related to post-thaw sperm viability, suggesting that this amino acid could be involved in oxidoreductase and oxidant reactions [66]. Interestingly, SP-tyrosine has also been found to contribute to the total antioxidant capacity of SP [67]. If these findings were confirmed in pigs, concentrations of phenylalanine and tyrosine in SP would also appear as exerting a beneficial effect upon sperm through regulation of reactive oxygen species (ROS) and could be used to predict fertility outcomes in porcine.

Finally, the relationship between gestation duration and concentration of SP-metabolites was also investigated, and whereas malonate showed higher levels in SP-samples from boars with longer gestation duration, fumarate exhibited lower levels in that group. However, further studies for fumarate validation should be conducted, as no information regarding the effect of this metabolite on sperm physiology or fertilizing ability has been published. On the other hand, malonate may have a double predictive value for both stillbirths per litter size and the estimation of gestation duration. Malonate is an intermediate metabolite of the Krebs cycle that inhibits ROS production via competition for succinate dehydrogenase [68, 69]. The present study identified a positive relationship between SP-malonate levels and both stillbirths per litter size and gestation duration. A similar negative influence of SP-malonate has also been found in humans, as infertile patients exhibited higher malonate levels than fertile controls [70]. On the other hand, malonate can act as protein post-translational modification [71]. Based on these findings, while no studies have been conducted to evaluate the influence of malonate on sperm physiology, it could be posited that a high SP-malonate concentration could: i) modify key proteins involved in gamete interaction or even embryo development, or ii) inhibit the Krebs cycle. In any case, the fact that malonate relates to two distinct *in vivo* fertility parameters reinforces its potential value as a predictor of AI outcomes in pig SP.

As aforementioned, the differences found between the results of the present research and those reported in other metabolomic studies conducted in pigs or in other species may be due to several factors: i) differences in the sensitivity of the metabolomic approaches; ii) variations in the preparation of samples; iii) the species-specific role of seminal metabolites in fertility; and iv) the use of non-comparable fertility parameters. For this reason, although -omics approaches are powerful tools, they should be used as a first steppingstone in the research of (in-)fertility biomarkers [10, 29, 72]. In effect, while the main strength of the present work is that a set of SP-metabolites has been proposed to predict AI outcomes, they should all be further validated using a higher number of individuals and other approaches to overcome the intrinsic limitations of-omics approaches. Following this, the measurement of metabolites in SP could be potentially used as an accurate fertility test to select boars before they are included in an AI-program. Moreover, future research needs to be conducted to assess i) the specific role of each SP-metabolite in male fertility, and ii) whether supplementing AI-extenders with specific metabolites can improve the fertility potential of semen doses.

Conclusions

The metabolite profiling of pig SP using NMR spectroscopy allowed the identification and quantification of 24 metabolites. The results evidenced that 13 of these

metabolites were related with AI outcomes, pointing out to putative *in vivo* fertility biomarkers. Specifically, lactate could be used as a farrowing rate indicator; carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate and glucose could predict litter size; citrate, creatine, phenylalanine, tyrosine and malonate would be biomarkers for the number of stillbirths per litter; and, finally, malonate and fumarate would anticipate the duration of gestation.

Abbreviations

AI: Artificial insemination; AUC: Area under the curve; NMR: Nuclear magnetic resonance; PCA: Principal components analysis; ROC: Receiver operating characteristic; ROS: Reactive oxygen species; SP: Seminal plasma; sPLS-DA: Sparse partial least square discriminant analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40104-021-00636-5.

Additional file 1 Supplementary Fig. 1 H-NMR (noesygppr1d) profile (600 MHz) from 0 to 8 ppm of pig seminal plasma.

Additional file 2 Supplementary Fig. 2 (A-D). Bayesian multiple logistic regression models for all the *in vivo* fertility parameters. The distribution of the coefficients (X axis) is depicted for each metabolite (Y axis). The coefficient distributions depict their effect on the model, as well as their associated uncertainties (credible intervals). Thus, changes in one unit on the coefficient value has a multiplicative effect on the log-odds of the prediction, equal to the value of the coefficient. Blue lines represent the 95% credible intervals, boxes show the 50% credible intervals, and dots are the distribution median.

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Authors' contributions

Conceptualization: YM-O, MY and IB; Methodology: YM-O, PF-L, PN and IB; Formal analysis and investigation: YM-O, PF-L, AD-B, JR-M, MY and IB; Writing - original draft preparation: YM-O; Writing - review and editing: PN, JR-M, JM, JR, MY and IB; Funding acquisition: JM, JR and MY; Supervision: IB and MY. All authors have read and agreed to the published version of the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethics approval and consent to participate

Ethical review and approval for animal participants was not required as samples were provided by the AI-Centre and authors did not manipulate any animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Discussion

Several roles have been attributed to SP, including the modulation of both male and female reproductive physiology. Yet, evidence supports that SP is not essential for successful fertilisation, as epididymal sperm used for *in vitro* fertilisation (IVF) and intra-cytoplasmatic sperm injection (ICSI) can produce embryos with great efficiency. In the case of humans, although IVF and ICSI procedures are of wide use to treat infertility, implantation rates are still below 40 %. Although a variety of factors contribute to implantation failure, the absence of SP in ART may be the biggest missing piece of the puzzle (Mcgraw et al., 2015). Accordingly, the **first Chapter of the present Dissertation aimed to investigate the extent to which SP can modulate *in vivo* fertility, and the potential molecular mechanisms underlying this regulation**.

The first work of the present Chapter assessed the effect of SP on *in vivo* embryo development. For this study, cattle were chosen as the most suitable animal model, because of the fertility reduction affecting the dairy industry. For many years, genetic selection of dairy cattle was only focused on increasing milk production, as this is the most financially lucrative area in this sector (Miglior et al., 2017). In spite of this, by the end of the 20th century, a decline in other relevant traits such as longevity, susceptibility to disease and, most relevant for the present work, calving rates, were identified (Pryce et al., 2004). In fact, a decline in conception rates of 1 % per year was observed (Boichard et al., 2002a, 2002b). Yet, since genetic selection for milk production traits only explains 30-50 % of the decline in the conception rates in dairy cattle (Grimard et al., 2006), the contribution of other factors should be further addressed.

While ≥ 70 % of ovulated oocytes are fertilised in lactating heifers after AI (Sartori et al., 2002), only 65 % of the resulting embryos are viable 5-6 days after AI (Santos et al., 2004). In fact, Humblot (2001) observed that early embryonic loss in pre-implantation embryo stages in dairy cows represents 20-45 % of all pregnancy failures, followed by late

embryonic/foetal loss (after day 16) (8-17 %) and late abortion (1-4 %) (Humblot, 2001). All these data suggest that the main cause of the decline in fertility rates in dairy cattle is not likely to be explained by oocyte fertilisation failure or late abortions, but by early embryonic death. In spite of this, it is not clear whether pregnancy loss in dairy cattle is due to failure in the establishment of an appropriate uterine environment for embryo development, intrinsic embryonic defects, or both. Focusing on the former, the female reproductive tract has been reported to be modified by seminal factors in many mammalian species, including mice, humans, pigs and cattle (Schjenken and Robertson, 2020). Against this background, **the first study of this Chapter aimed to elucidate the effect of SP exposure on *in vivo* pre-implantation embryo survival and development**. To this end, heifers in standing oestrus were either mated with vasectomised bulls (which only deposited SP upon ejaculation; SP exposed) or left unmated (control). Then, *in vitro* produced embryos were transferred 7 days after mating to both groups. Heifers were slaughtered and embryos were recovered at day 14 to assess conceptus survival, developmental stage and gene expression. In addition, CL was also evaluated in terms of volume at day 7, as well as weight and volume at day 14.

The results of this first study showed no differences in the volume or weight of CL, either at day 7 or day 14, which could be interpreted as no effect of SP on ovarian physiology in cattle. Previous studies conducted in mice (Gangnuss et al., 2004) and pigs (O'Leary et al., 2006) reported that exposure to SP (by mating in mice, or by infusion into the uterus in pigs) induced an increase of macrophage recruitment into the ovulatory follicle (Gangnuss et al., 2004; O'Leary et al., 2006). Yet, while this recruitment was not detected to affect CL physiology in mice (Gangnuss et al., 2004), which would resemble to the findings reported here, an increase in steroidogenesis in response to SP exposure in pigs was observed (O'Leary et al., 2006). The discrepancy between cattle and pigs could be explained by the different time-points selected for analysis. In cattle, an experiment

assessing the effects of the exogenous administration of P4 on the modulation of CL behaviour between days 3 and 7 post-ovulation determined that there was no variation in the CL weight at day 14 (O'Hara et al., 2014). These data would agree with the results of the present work, as no differences in the volume and weight of CL were observed either at day 7 or day 14. One, however, should not discard macrophage recruitment during days 3 to 6, which could lead to faster CL growth and higher peripheral levels of P4 before day 7. Unfortunately, the time-points chosen for this experiment did not allow testing this hypothesis. Conversely, it cannot be discarded that bovine SP might not be able to affect ovarian physiology, as occurs in mice (Gangnuss et al., 2004). Finally, it is worth mentioning that the potential species-specific differences in the role of SP in ovarian physiology could be related to the particular reproductive strategies of each species.

Studies in mice (Bromfield et al., 2014) and humans (Tremellen et al., 2000) previously suggested that the presence of SP can improve pregnancy rates. In spite of this, in the present study, no differences in the embryo recovery rate (and, therefore, embryo survival) were observed between SP-treated and control groups, harvesting, at day 14, the exact 50 % of the embryos transferred to both groups at day 7, values that are in line with the usual recovery in cattle (Betteridge et al., 1980; Diskin and Morris, 2008). These results are consistent with other investigations showing that intrauterine SP infusion does not improve pregnancy rates in cattle (Odhiambo et al., 2009; Pfeiffer et al., 2012; Ortiz et al., 2019). Interestingly, a similar experiment carried out in pigs also found equal embryo viability at early stages of pre-implantation between SP-treated and control groups (Martinez et al., 2019). Again, the potential differences between mice, humans, pigs and cattle could respond to species-specific effects of SP on the uterine environment, thus affecting embryo survival.

Despite the lack of differences in embryo recovery rates, a trend towards longer conceptuses (average conceptus length of ≥ 4 mm) in heifers exposed to SP in comparison to the control group was detected. This last result was confirmed when embryo length was analysed on a morphology basis (ovoid (0.5-4 mm), tubular (days 5-19 mm) and filamentous (> 20 mm); (Ribeiro et al., 2016)). Specifically, the results showed that longer filamentous embryos from SP-exposed heifers were recovered. Similarly, in pigs, embryos at later preimplantation developmental stages (day 6) were retrieved from SP-infused uterus (Martinez et al., 2019). In any case, as conceptus length is related to the elongation of extraembryonic tissues rather than to the growth of the ICM (Bolmberg et al., 2008), the greater conceptus length observed herein should not be directly attributed to better embryo development. Consequently, embryo gene expression for several developmental markers was then evaluated in this first work.

Embryo gene expression was found to differ between groups, particularly in the expression of Calmodulin 1 (*CALM1*), Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*), Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxyterminal (*CITED1*), Dihydrolipoamide Dehydrogenase (*DLD*), Heterogeneous Nuclear Ribonucleoprotein D Like (*HNRNPDL*) and Transforming Growth Factor Beta 3 (*TGFB3*). Most of the differences in gene expression between treatments were observed when comparing long filamentous conceptuses, rather than when looking into other embryo morphologies. Interestingly, expression levels of these genes differed between short and long conceptuses only in the control group, whereas these disparities were not detected in embryos recovered from SP-primed uteri. All these findings suggest that the indirect regulation of specific signalling pathways in response to SP exposure only occurs in very advanced embryos, possibly because it is not until embryo hatching and implantation that uterine receptivity becomes crucial. Seminal factors have been widely reported to be able to modify uterine gene expression in species such as humans (Sharkey et al., 2012b; Chen et al., 2014), rodents

(Song et al., 2016), pigs (O'Leary et al., 2004; Martinez et al., 2019) and cattle (Elweza et al., 2018; Ibrahim et al., 2019; Recuero et al., 2020). Although many signalling pathways have been described to be influenced by seminal factors, those related to immune response, cell adhesion and development are of particular interest for the present work. Indeed, besides the remodelling of the uterine environment to promote implantation, the secretion of embryotrophic factors in response to seminal factors could also explain the positive effect of SP on embryo development in cattle (Schjenken and Robertson, 2020). Noticeably, this was previously reported in pigs, where SP was found to be able to up-regulate genes related to embryo development and pregnancy progression (Martinez et al., 2020).

After characterising how SP was able to modulate *in vivo* embryo development, this Dissertation investigated whether SP biomolecules, in particular proteins (AKR1B1) and metabolites, could influence *in vivo* fertility outcomes. In both studies, the pig was chosen as the most appropriate animal model because of: i) the large SP volume retrieved from pig ejaculates compared to other mammalian species, such as humans, mice or even cattle; and ii) the physiological, anatomical and genomic similarities to humans (Archibald et al., 2010; Zigo et al., 2020).

As explained in detail in Section 5.1.1 of the Introduction, many efforts have been made towards the identification of SP proteins able to explain what drives reproductive success. One of those studies reported that the AKR1B1 present in SP could be a potential *in vivo* fertility biomarker in pigs (Pérez-Patiño et al., 2018). This protein is involved in the polyol pathway, catalysing the conversion of glucose into sorbitol (Avancini and Rossing, 2015), and the reduction of carbonyl-containing metabolic compounds, acting as a detoxification enzyme (Srivastava et al., 2005). Interestingly, it has been suggested to influence female and male reproductive events in several mammalian species, including humans (Bresson et al., 2011), cattle (Frenette et al., 2004; Girouard et al., 2009), rats

(Kobayashi et al., 2002), mice (Jagoe et al., 2013), sheep (Yang et al., 2019) and pigs (Steinhauser et al., 2016). Considering its relevance in reproductive physiology, **the second work of Chapter 1 carried out an in-depth characterisation of AKR1B1 alongside the male reproductive tract and investigated the potential involvement of SP-AKR1B1 on sperm function and, consequently, *in vivo* fertility.** To this end, three experiments were set: i) the determination of whether SP-AKR1B1 is present in ejaculate portions/fractions, assessed through enzyme-linked immunosorbent assay (ELISA); ii) the analysis of AKR1B1 expression in male reproductive organs through immunohistochemistry and immunoblotting assays; and iii) the evaluation of the relationship between SP-AKR1B1 levels and sperm quality (including sperm motility, viability and morphology) and functionality (including intracellular H_2O_2 production by viable sperm, and acrosome integrity and plasma membrane lipid disorder in viable sperm) parameters.

The porcine ejaculate is emitted in distinct fractions that differ in terms of sperm concentration and SP origin and composition: pre-ejaculate fraction, SRF and post-SRF. In addition, the SRF can in turn be divided into two distinct portions based on sperm concentration, SRF-P1 and SRF-P2. Briefly, the SRF-P1 contains most of the ejaculated sperm and its SP is essentially composed by epididymal secretions, whereas that of SRF-P2 mainly originates from the epididymis and the prostate. Regarding the post-SRF, the SP is mostly produced by seminal vesicles (Einarsson, 1971; Saravia et al., 2009; Rodriguez-Martinez et al., 2011). Interestingly for this work, ejaculate portions have been described to have different traits in terms of sperm physiology. For instance, sperm contained in the SRF-P1 seem to hold the best traits (Sellés et al., 2001; Peña et al., 2003; Rodríguez-Martínez et al., 2005; Saravia et al., 2009; Alkmin et al., 2014; Li et al., 2018). Taking these differences between ejaculate fractions/portions into account, the analysis of their composition can be used to estimate the physiological relevance of specific components for sperm functionality. For this reason, the first experiment of the second work of Chapter 1 aimed to determine the

concentration of SP-AKR1B1 in the different ejaculate fractions/portions. The results showed that AKR1B1 was present in the SP of all ejaculate fractions/portions, with a higher concentration of the protein in the SP of SRF-P2 and PSRF. This was further supported by the fact that AKR1B1 was detected, through immunoblotting and immunohistochemistry assays, in the testis, epididymis and all accessory sex glands, except the bulbourethral ones. These results were in agreement with observations in male rats, in which AKR activity was detected throughout the male reproductive tract, notwithstanding bulbourethral glands were not analysed (Kobayashi et al., 2002; Iuchi et al., 2004). Furthermore, other studies conducted in cattle and pigs demonstrated the presence of AKR1B1 in seminal vesicles (Samuels et al., 1962; Westfalewicz et al., 2017). As the SRF-P1, which contains most of the ejaculated sperm, had lower amounts of AKR1B1, one could speculate that SP-AKR1B1 might not play an essential role in sperm physiology.

Based on the findings mentioned in the previous paragraph, the potential relationship between SP-AKR1B1 concentration in the entire ejaculate and sperm quality and functionality was investigated at 0 h and after 72 h of liquid storage at 17 ºC. Remarkably, no relationship between SP-AKR1B1 concentration and any of the sperm quality and functionality parameters assessed (i.e., sperm concentration, sperm with normal morphology, total and progressive motile sperm, viable sperm, viable sperm with high intracellular ROS, viable sperm with a damaged acrosome and viable sperm with high membrane lipid disorder), was observed at any of the timepoints. The main functions of AKR1B1 in male reproductive physiology reported thus far are related to epididymal sperm maturation in cattle (Frenette et al., 2003) and mice (Jagoe et al., 2013), and sperm capacitation in pigs (Katoh et al., 2014). In consideration of all these findings, particularly the lack of influence from SP-AKR1B1 on sperm quality and functionality parameters found in this work, together with the fact that the highest SP-AKR1B1 concentration was not found in the SRF-P1, it is

reasonable to surmise that SP-AKR1B1 does not play a major role on sperm physiology.

The explanation for the positive relationship between SP-AKR1B1 and *in vivo* fertility outcomes reported in pigs (Pérez-Patiño et al., 2018) could thus reside on the function of this protein in the female reproductive tract. Uterine AKR1B1 has been reported to act as a prostaglandin synthase, particularly enhancing the production of PGF2α and PGE2 in the endometrium of humans (Bresson et al., 2011), cattle (Madore et al., 2003) and pigs (Seo et al., 2014). Considering that pig semen is deposited into the cervix, it is reasonable to hypothesise that SP-AKR1B1 could join the uterine AKR1B1 enhancing PG production. Since PGF2α is a luteolytic factor (Jensen et al., 1987) and PGE2 is a pro-luteal factor (Niringiyumukiza et al., 2018), it is likely that SP-AKR1B1 would preferentially boost the production of PGE2 to further promote embryo implantation and development. Yet, although this hypothesis could potentially explain the observed positive relationship with *in vivo* fertility, a direct effect of SP-AKR1B1 on embryo development should not be discarded if AKR1B1 from SP was transferred to sperm. Interestingly, bovine AKR1B1 has been reported to be associated to epididymal EVs (Frenette et al., 2006) and, in humans, seminal EVs have been found to contain AKR (Zhang et al., 2020). In pigs, however, whether this protein is also present in the cargo of seminal EVs and whether it is involved in fertilisation or embryo development were not previously interrogated. The implication of the AKR1B1 contained in sperm on oocyte fertilisation and embryo development was investigated in Chapter 2.

Finally, the potential involvement of SP-metabolite composition on *in vivo* reproductive success was assessed in the third work of Chapter 1. The emergence of high-throughput technologies, including metabolomics, has favoured the characterisation of metabolites, the end-products of downstream events of cellular signalling pathways (Goodacre et al., 2004). In the last years, metabolites have been proposed as markers of patho- and

physiological reproductive processes. In particular, certain metabolites of SP were identified as (in)fertility biomarkers in several mammalian species, including humans (Hamamah et al., 1993; Qiao et al., 2017; Mumcu et al., 2020; Xu et al., 2020), pigs (Zhang et al., 2021) and cattle (Kumar et al., 2015; Velho et al., 2018; Talluri et al., 2022). Particularly in pigs, Zhang et al. (2021) compared the SP metabolome profile between boars with high and low conception rates after AI using ultra-high performance LC-Q-TOF-MS, identifying some SP-metabolites (such as Pro-Asn, Ile-Tyr, and D-Biotin) as potential fertility biomarkers (Zhang et al., 2021). Yet, the work included in this Dissertation aimed to provide a wider picture of the effect of SP-metabolites on *in vivo* fertility outcomes, including other parameters such as farrowing rate, litter size, stillbirths per litter and duration of gestation. Thus, **the aim of the third work of Chapter 1 was to evaluate the relationship between SP-metabolites and reproductive success using NMR spectroscopy**. Since this was a first steppingstone in the identification of potential *in vivo* fertility biomarkers in pig SP, an untargeted approach was followed. To this end, three ejaculates per boar were collected every four months, from which SP was separated for metabolomic analysis. In parallel, a total of 1,525 weaned multiparous sows (1–7 litters produced) were inseminated throughout a year. Fertility outcomes were recorded for each AI-boar, including: (1) farrowing rate (percentage of inseminated sows that farrowed), (2) litter size (total number of piglets born per litter), (3) number of stillbirths per litter, and (4) duration of pregnancy (days). A model described by Broekhuijse et al. (2012) was used to isolate the direct boar effect on each *in vivo* fertility parameter (Broekhuijse et al., 2012). Finally, boars were divided into two groups (above- and below- the median) for each of these parameters before statistical analysis.

The work led to the identification and quantification of 24 metabolites in pig SP, mainly categorised into amino acids, alcohols, saccharides, salts and other organic compounds (including carnitine, creatine, creatine-phosphate, hypotaurine, myo-inositol, sn-glycero-3-

phosphocholine and trimethylamine N-oxide). All the identified metabolites were present in samples, so that no qualitative differences between boars were observed. Following this, the relationship between the levels of specific metabolites and *in vivo* fertility parameters was investigated. The main findings were: i) the concentration of lactate in SP was related to farrowing rates; ii) concentrations of carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate and glucose in SP were associated with litter sizes; iii) concentrations of citrate, creatine, malonate, phenylalanine and tyrosine in SP were related to stillbirths per litter; and iv) concentrations of malonate and fumarate in SP were associated to the duration of gestation. In addition, their potential as biomarkers was tested through ROC analysis. Interestingly, the previous literature from the SP metabolites identified as potential *in vivo* fertility biomarkers suggested most of them were able to influence sperm physiology in terms of sperm metabolism, sperm motility or sperm capacitation. Only the most relevant relationships are discussed below.

Regarding sperm metabolism, lactate was found to negatively correlate with farrowing rate, meaning that the highest lactate concentrations in SP were found in samples from boars with a negative farrowing rate deviation. Lactate is one of the main non-monosaccharide substrates used by cells for energy production. In particular, sperm from several mammalian species, including bulls (Menezes et al., 2019), stallions (Darr et al., 2016), men (Rodriguez-Gil, 2006) and boars (Rodriguez-Gil, 2006; Paventi et al., 2015) are known to be able to use lactate as an energy source. Particularly in pigs, sperm have been reported to metabolise lactate via lactate dehydrogenase (LDH; Medrano et al., 2006). This, together with the findings presented here would point out that boars classified as having a positive farrowing rate deviation could better metabolise lactate for energy production, thereby leading to lower SP-lactate concentration. On the other hand, concerning glucose, results showed that glucose concentrations were lower in the SP of boars with an increased litter size deviation than in that

of boars with a decreased litter size. Glucose is one of the main monosaccharides used by mammalian sperm for energy production (Albarracín et al., 2004; Rodriguez-Gil, 2006). The most feasible explanation for such a finding would be that sperm of boars with a reduced litter size would consume less glucose from their SP; thus, glucose would extracellularly accumulate and would not be used for energy production. These data would suggest an influence of sperm metabolism on reproductive success. Moreover, because when conducting this study, it was still unclear which metabolic pathway is preferentially used by pig sperm (Miki et al., 2004; Nesci et al., 2020), this was further addressed in Chapter 2.

Metabolites such as carnitine, sn-glycero-3-phosphocholine, creatine and phenylalanine are also of particular interest for this Dissertation. Particularly, while carnitine and sn-glycero-3-phosphocholine were found to be positively related to litter size, creatine and phenylalanine were seen to be negatively related to stillbirths per litter. Interestingly, these metabolites have been reported to regulate sperm physiology. For instance, both carnitine (Jeulin and Lewin, 1996; Chavoshi Nezhad et al., 2021) and creatine (Umehara et al., 2018; Nasrallah et al., 2020) are known to positively influence sperm motility and viability, which ultimately translates into improved seminal characteristics (Kozink et al., 2004; Yeste et al., 2010). On the other hand, sn-glycero-3-phosphocholine (Zanetti et al., 2010) and phenylalanine (Houston et al., 2015) have been described to modulate sperm capacitation and the acrosome reaction, essential processes for oocyte fertilisation. Thus, their implication in particular traits of sperm function is likely to explain why they influence fertility outcomes positively.

Although the literature indicates that these SP metabolites mainly exert an effect on sperm, a role in shaping the female environment cannot be precluded. Indeed, one could hypothesise that they could potentially account for the activation of the female immune response or even the

secretion of embryotrophic/embryotoxic factors. In effect, to the best of the author's knowledge, most of the efforts made to identify female tract modulators have been directed to SP proteins. In view of the relationship between SP metabolites and *in vivo* fertility outcomes, future studies should also address whether specific metabolites contained in the SP interact and modulate the female reproductive system.

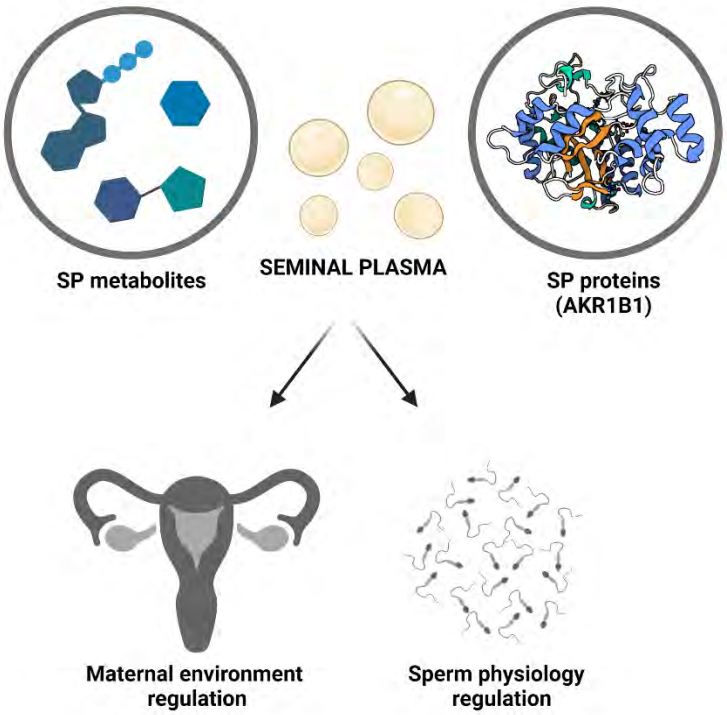

***Figure 5.* Seminal plasma (SP) *in vivo* fertility modulatory mechanisms.** Schematic representation of the main findings of Chapter 1 of the present Dissertation. Created with BioRender.

In summary, the results presented here indicate that SP has an impact on embryo development, probably through the regulation of both the uterine environment and sperm physiology (**Figure 5**). Certainly, the present Chapter determined that the molecular mechanisms underlying the function of SP on sperm and the female reproductive tract probably involve proteins, such as AKR1B1, and metabolites. Yet, the exact pathways via which they might be conditioning fertility, some of them hypothesised in this Dissertation, remain unknown and should be interrogated in the future.

Chapter 2

Sperm factors regulate fertilisation and embryo development

Paper IV

Aldose Reductase B1 in Pig Sperm Is Related to Their Function and Fertilizing Ability.

Yentel Mateo-Otero, Jordi Ribas-Maynou, Ariadna Delgado-Bermúdez, Marc Llavanera, Sandra Recuero, Isabel Barranco, Marc Yeste.

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Aldose Reductase B1 in Pig Sperm Is Related to Their Function and Fertilizing Ability

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Aldose reductase B1 (AKR1B1) has been reported to participate in the modulation of male and female reproductive physiology in several mammalian species. In spite of this, whether or not AKR1B1 could be related to sperm quality, functionality and fertilizing ability is yet to be elucidated. The present study, therefore, aimed to investigate: i) the presence of AKR1B1 in epididymal and ejaculated sperm; ii) the relationship between the AKR1B1 present in sperm and the physiology of the male gamete; iii) the liaison between the relative content of AKR1B1 in sperm and their ability to withstand preservation for 72 h; and iv) the potential link between sperm AKR1B1 and *in vitro* fertility outcomes. Immunoblotting revealed that AKR1B1 is present in both epididymal and ejaculated sperm with a similar relative content. Moreover, the relative levels of AKR1B1 in sperm (36 kDa band) were found to be negatively related to several kinematic parameters and intracellular calcium levels, and positively to the percentage of sperm with distal cytoplasmic droplets after storage. Finally, AKR1B1 amounts in sperm (36 kDa band) were negatively associated to fertilization rate at two days post-fertilization and embryo development at six days post-fertilization. The results of the present work suggest that AKR1B1 in sperm is probably acquired during maturation rather than at ejaculation and could play a role in that process. Moreover, AKR1B1 seems to be related to the sperm resilience to preservation and to their fertilizing capacity, as lower levels of the 36 kDa band (putative inactive form of this protein) result in better reproductive outcomes.

Keywords: aldose reductase B1, AKR1B1, epididymal maturation, ejaculated sperm, sperm physiology, *in vitro* fertilization (IVF)

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INTRODUCTION

Predicting fertility remains a major challenge for reproductive biology in mammals (1), that is why a significant number of studies have focused on uncovering male fertility biomarkers in the last years (2–4). In this context, proteomic characterization of both seminal plasma [SP; (5, 6)] and sperm (7) has gained much relevance. Pérez-Patiño et al. (2018) performed an in-depth proteomic analysis of pig SP revealing that specific proteins were related to sperm fertilizing ability (8). Among these proteins, these authors identified Aldose Reductase B1 (AKR1B1 or ALR2), which is a NADPH-dependent enzyme

that belongs to the aldo-keto reductase protein superfamily (9, 10) and is positively related to *in vivo* fertility outcomes (8).

Aldose Reductase B1 is the most studied aldose reductase and participates in both the polyol pathway and the detoxification of carbonyl compounds in many cells and tissues (11–13), including the male genital tract (14, 15). This protein has been reported to be involved in both male and female reproductive physiology in several mammalian species, including humans (16), cattle (17, 18), rats (15), sheep (19) and pigs (8, 20, 21). Focusing on the male, while AKR1B1 in SP has been reported to exert a positive effect on *in vivo* fertility outcomes in porcine (8), it does not seem to influence sperm physiology in terms of sperm survival and motility, intracellular H_2O_2 levels, acrosome integrity and plasma membrane stability (21). Not only is AKR1B1 present in SP but also in sperm, where it appears to be involved in epididymal maturation through the polyol pathway for fructose production (17, 22, 23). In addition, AKR1B1 is activated during sperm capacitation and modulates sperm motility, probably through balancing reactive oxygen species (ROS) production (24). Yet, whether does sperm AKR1B1 modulate other sperm functional parameters or influence the sperm ability to fertilize the oocyte has not been investigated.

Although aldose reductases have been identified in bovine (17, 25), equine (26) and porcine sperm (24), the origin of this protein in mature sperm cells is still unknown. In this regard, while translation during spermatogenesis could be a possibility, no previous study has confirmed if the relative content of AKR1B1 is higher in ejaculated than in epididymal sperm. In bovine sperm, the relative amount of this protein has been found to increase along the epididymal transit (17, 25), probably due to the integration of epididymosomes at the cauda (25). In addition, bovine prostasomes have been reported to contain AKR1B1 (22). For this reason, determining whether the relative content of AKR1B1 is higher in ejaculated than in epididymal sperm would provide further evidence on the aforementioned contribution of the extracellular vesicles present in SP.

Considering the relevance of AKR1B1 as a vital protein in mammalian reproductive physiology, the main aim of the present study was to determine the potential involvement of sperm AKR1B1 in both sperm physiology and fertility outcomes using the pig as a model. The following specific objectives were set: (1) to identify the presence of AKR1B1 in epididymal and ejaculated sperm in order to elucidate whether this protein is acquired from SP upon ejaculation; (2) to assess the relationship between sperm AKR1B1 and the physiology of male gametes; (3) to determine whether sperm AKR1B1 is related to the sperm resilience to preservation in liquid storage; and (4) to evaluate the relationship between the relative content of AKR1B1 in sperm and their *in vitro* fertilizing ability.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all reagents used in this study were of analytical grade and acquired from Sigma (Merck, Darmstadt, Germany). Fluorochromes were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Animals and Samples

Semen samples were acquired from a local artificial insemination (AI) center (Grup Gepork S.L., Masies de Roda, Spain), which follows the ISO certification (ISO-9001:2008). The AI center performed all the procedures that involved animals in accordance with the EU Directive 2010/63/EU for animal experiments; the Animal Welfare Law issued by the Regional Government of Catalonia, Spain; and the current regulation on Health and Biosafety issued by the Department of Agriculture, Livestock, Food and Fisheries, Regional Government of Catalonia, Spain. Ejaculates from healthy and sexually mature Pietrain boars (1-3 years old) were collected using the hand-gloved method. Samples were immediately diluted to a final concentration of 33×10^6 sperm/mL using a commercial extender (Vitasem LD, Magapor S.L., Zaragoza, Spain) and stored at 17°C until use.

For epididymal sperm samples, four healthy boars were slaughtered in a commercial slaughterhouse for genetic replacement reasons. Once slaughtered, the epididymis was collected and transported in insulated container at 5°C to our laboratory. Epididymal sperm were then flushed by placing a needle in the ductus deferens and retrogradely infusing air. The luminal fluid was collected at a section between corpus-cauda limit. The fluid from the two epididymes of each boar was pooled and was microscopically evaluated to confirm that more than 75% of sperm were viable (SYBR-14/PI staining).

No animal was manipulated by the authors, as ejaculated semen samples were acquired from a local farm (AI-center) and the abattoir donated the epididymis of boars that were sacrificed for culling reasons. No permission from an Ethics Committee was, therefore, required.

Experimental Design

First, the presence of AKR1B1 was assessed in epidydimal and ejaculated sperm with the objective to elucidate whether this protein is acquired during ejaculation from SP. To this end, the epididymis (n=4) was flushed and the epididymal fluid was centrifuged twice (3,000×g and room temperature for 5 min) to harvest epididymal sperm. The resulting pellet was lysed to determine the levels of AKR1B1 in epididymal sperm with the Western Blot assay. On the other hand, ejaculated semen samples (n=4) were centrifuged twice (3,000 ×g and room temperature for 5 min), and the sperm pellet was lysed to determine AKR1B1 levels in ejaculated sperm also through Western Blotting.

Second, the relationship between sperm AKR1B1 levels and several sperm quality and functionality parameters were investigated. For these experiments, commercial semen samples (n=15) were split into three aliquots. The first aliquot was used to assess initial sperm quality and functionality parameters immediately after semen samples arrived at the laboratory (0 h). The second aliquot was used to evaluate sperm quality and functionality parameters after liquid storage at 17°C for 72 h. Finally, the third aliquot was centrifuged twice

(3,000 ×g and room temperature for 5 min) to obtain the pellet, which was stored at -80°C until the relative content of AKR1B1 in sperm was determined.

Third, the relationship between sperm AKR1B1 and fertilizing ability was evaluated through *in vitro* fertilization using the semen samples from 24 boars (n = 24).

Western Blot

The immunoblotting assay was used to determine the presence of AKR1B1 in ejaculated and epididymal sperm and to quantify the relative AKR1B1 content in the different sperm samples. In all cases, proteins were extracted from samples using xTractor lysis buffer (xTractor® Buffer; Takara Bio, Mountain View, CA, USA), supplemented with 1% protease inhibitor, 0.1 M phenylmethylsulfonyl fluoride and 700 mM orthovanadate. Samples were incubated for 30 min on ice, with vortexing every 5 min, and then sonicated three times with five pulses. Once sonicated, they were centrifuged at 12,000 ×g and 4°C for 20 min. Supernatants were collected in siliconized Eppendorf tubes and stored at -80°C until protein quantification. Protein quantification was carried out in triplicate using a detergent compatible (DC) method (Bio-Rad; Hercules, CA, United States) and an Epoch Microplate Spectrophotometer (BioTek®; Winooski, VT, USA). All samples were adjusted to a final concentration of 2.5 μg/μL of total protein with the lysis buffer.

A total of 20 μg of protein was mixed (1:1, v:v) with 4× Laemmli Reducer supplemented with 5% (v:v) β-mercaptoethanol (Bio-Rad) and subsequently heated at 95°C for 7 min. A final volume of 16 μL was loaded onto 8-16% gradient gels (Mini-Protean®, TGX Stain-Free™ Precast Gels, Bio-Rad), and electrophoresis was conducted at 150 V for 2 h. Afterwards, proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) using a Trans-Blot® Turbo™ system (Bio-Rad). For total protein quantification, membranes were exposed to 180 sec of UV and then read using a G:BOX Chemi XL system (SynGene; Frederick, MD, USA). Following this, membranes were blocked using blocking buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween-20 and 5% bovine serum albumin [BSA]; pH = 7.3) for 1 h under agitation. Next, membranes were incubated with an AKR1B1 primary antibody (1:1,000 diluted in blocking buffer; ref. HPA026425, Prestige Antibodies, Merck; Germany) at 4°C overnight with agitation. In order to determine the specificity of the primary antibody, one membrane was co-incubated with the AKR1B1 blocking peptide (ref. APREST77862, Prestige Antibodies, Merck) 20 times more concentrated than the antibody. On the next day, membranes were washed thrice with 1× TBS Tween 20 (10 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20; pH = 7.3) before incubation with an anti-rabbit secondary antibody conjugated with HRP (1:2,000 diluted in blocking buffer; ref. P0448, Merck) for 1 h with agitation. Finally, prior to visualization of bands, blots were washed six times (5 min each) with 1× TBS Tween 20. Detection was performed after incubation of membranes with a chemiluminescent substrate (Immobilon™ Western Detection Reagents, Millipore, United States) for 5 min, and scanning with a G:BOX Chemi XL 1.4 system (Syngene, Cambridge, UK). In all blots, two specific bands (36 kDa and 80 kDa) were observed.

Image Studio Lite v5.2.5 software (LICOR, Lincoln, NE, USA) was used for image analysis of the resulting blots. For each blot, the background level was subtracted from the density of 36 kDa and ~80 kDa bands. Moreover, each band was normalized by dividing its value with background levels. Finally, the resulting band intensity was also divided with the total protein quantity of each sample. Three technical replicates per sample were evaluated.

While the molecular weight of the monomeric AKR1B1 form is 36 kDa, the identity of the ~80 kDa band was investigated through an additional immunoblotting assay. Two pools (5 ejaculates each; one ejaculate per boar) of sperm lysates were incubated (1:1, v:v) with 16 M urea at room temperature for 1 h. Next, samples were subjected to electrophoresis and Western Blot following the previously described protocol.

Evaluation of Sperm Quality and Functionality

Sperm Motility

A computer-assisted sperm analysis (CASA) system was used to assess sperm motility using an Olympus BX41 microscope (Olympus; Tokyo, Japan) with a negative phase contrast field (Olympus 10 X 0.30 PLAN objective, Olympus) connected to a computer running the ISAS software (Integrates Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). Before motility analysis, samples were incubated at 38°C for 15 min. To examine sperm motility, 3 μL of each sample was placed into a prewarmed (38°C) Leja20 counting chamber (Leja Products BV; Nieuw-Vennep, The Netherlands). Two technical replicates, with at least 500 sperm per replicate, were counted.

Eight sperm velocity parameters were recorded: straight line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), percentage of linearity (LIN), percentage of straightness (STR) and motility parameter wobble (WOB). Total motility and progressive motility were also recorded. Sperm were considered motile when VAP was ≥ 10 μm/s, and progressively motile when STR was over 45%.

Sperm Morphology

Sperm morphology was examined in semen samples diluted (1:1, v:v) with 0.12% formaldehyde saline solution (PanReac AppliChem; Darmstadt, Germany); a phase-contrast microscope at 1,000× magnification was used (Nikon Labophot; Nikon; Tokio, Japan). A total of 200 sperm cells were counted and those without morphology aberrations were considered as normal. Moreover, secondary alterations including sperm with proximal and distal cytoplasmic droplets and sperm with folded tails were recorded (27).

Flow Cytometry Assessment

Sperm viability, intracellular calcium levels and acrosome membrane integrity were assessed using a Cytoflex cytometer (Beckman Coulter; Fullerton, CA, USA). Semen samples were diluted (4×10^6 sperm/mL) in phosphate buffered saline (1× PBS) prior to staining sperm. Briefly, sperm viability was evaluated

using SYBR-14 and propidium iodide (PI), where SYBR-14 stains the nuclei of all sperm and PI only stains those of sperm that have lost their plasma membrane integrity (28). Intracellular calcium levels were evaluated through Fluo3/PI staining (29). Fluo3-AM is a non-fluorescent, non-polarized membrane-permeable dye that exhibits green fluorescence when binds to calcium (30). Acrosome membrane integrity was assessed using fluorescein-conjugated peanut agglutinin (PNA), which is a lectin that binds to the inner leaflet of the outer acrosomal membrane (31). Finally, mitochondrial membrane potential was evaluated using 5,5',6,6'-tetrachloro-1,1',3,3'tetraethyl-benzimidazolylcarbocyanine iodide (JC-1), that aggregates in the presence of high mitochondrial membrane potential and emits orange fluorescence (32). Throughout all the experiment, flow rate, laser voltage and sperm concentration remained unchanged. Forward scatter (FSC) and side scatter detectors (SSC) were utilized to identify sperm cells from debris events. For each sample, three technical replicates containing at least 10,000 sperm were evaluated. The CytExpert software (Ver, 2.3, Beckman Coulter) was used to analyze flow cytometry data.

Sperm viability was evaluated using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA), following the protocol of Garner and Johnson (1995) with minor modifications (28). Briefly, sperm were stained with SYBR-14 (final concentration: 32 nM) and PI (final concentration: 7.5 μM) at 38°C in the dark for 15 min, and subsequently analyzed with a CytoFLEX cytometer (Beckman Coulter; Fullerton, CA, USA). SYBR-14 fluorescence was detected by the fluorescein isothiocyanate (FITC) channel (525/40), and that of PI through the PC5.5 channel (690/50). Both fluorochromes were excited with a 488-nm laser, and no spill compensation was applied. The percentage of viable sperm ($SYBR\text{-}14^+/PI^-$) was recorded and used for the subsequent statistical analysis.

Sperm intracellular calcium levels were evaluated following the protocol set by Harrison et al. (1993, 29). Briefly, sperm were double stained with a solution of Fluo3-AM (final concentration: 1.2 μM) and PI (final concentration: 5.6 μM) at 38°C for 10 min. Fluorescence from Fluo3 was detected through the FITC channel (525/40). Four sperm populations were identified in dot-plots: i) viable sperm with low levels of intracellular calcium ($Fluo3^-/PI^-$); ii) viable sperm with high levels of intracellular calcium ($Fluo3^+/PI^-$); iii) non-viable sperm with low levels of intracellular calcium ($Fluo3^-/PI^+$); and iv) non-viable sperm with high levels of intracellular calcium ($Fluo3^+/PI^+$). The percentage of viable sperm with high intracellular calcium ($Fluo3^+/PI^-$) and the mean of Fluo3 fluorescence intensity per sperm were recorded and used for the subsequent statistical analysis.

Acrosome membrane integrity was evaluated using PNA-FITC/PI following the protocol set by Nagy et al. (2003, 31). Briefly, sperm were double stained with PNA conjugated with FITC (final concentration: 1.2 μM) at 38°C for 5 min in the dark. Next, sperm were stained with PI (final concentration: 5.6 μM) at 38°C for 5 min in the dark. PNA-FITC was detected by the FITC channel (525/40). Four sperm populations were observed: i) viable membrane-intact sperm ($PNA\text{-}FITC^-/PI^-$); ii) non-viable sperm having a damaged plasma membrane and an outer acrosome membrane not completely intact ($PNA\text{-}FITC^+/PI^+$); iii) non-viable sperm with a damaged plasma membrane and a completely lost outer acrosome membrane ($PNA\text{-}FITC^-/PI^+$); iv) viable sperm with a damaged plasma membrane ($PNA\text{-}FITC^+/PI^-$). The percentage of viable sperm with an intact acrosome membrane ($PNA\text{-}FITC^-/PI^-$) was recorded and used for the subsequent statistical analysis.

Mitochondrial membrane potential was evaluated with JC-1 following the protocol from Ortega-Ferrusola et al. (2008, 32). In brief, samples were incubated with JC-1 (final concentration: 750 nmol/L) at 38°C for 30 min in the dark. High mitochondrial membrane potential causes JC-1 aggregation, which results in orange fluorescence emission collected through the PE channel. On the contrary, JC-1 remains as a monomer in the presence of low mitochondrial membrane potential, emitting green fluorescence that is collected through the FITC channel. Three sperm populations were, therefore, distinguished: i) sperm with low mitochondrial membrane potential (green-stained); (ii) sperm with high mitochondrial membrane potential (orange-stained); and (iii) sperm with heterogeneous mitochondria (green and orange-stained in the same cell).

Oocyte Maturation and *In Vitro* Fertilization

Ovaries were obtained from pre-pubertal gilts slaughtered at a local abattoir (Frigoríficos Costa Brava; Riudellots de la Selva, Girona) and transported to the laboratory in 0.9% NaCl supplemented with 70 μg/mL kanamycin at 38°C. Cumulus-oocyte complexes (COC) were collected from follicles and only COCs with complete and compact cumulus mass were selected and washed in Dulbecco's PBS (Gibco, ThermoFisher) supplemented with 4 mg/mL of BSA.

The maturation medium used was TCM-199 (Gibco) supplemented with 0.57 mM cysteine, 0.1% (w:v) PVA, 10 ng/mL EGF, 75 μg/mL of penicillin-G potassium, and 50 μg/mL of streptomycin sulfate. Groups of 40-50 COCs were transferred to a four-well multi-dish (Nunc, ThermoFisher; Waltham, MS, USA) containing 500 μL of pre-equilibrated maturation media supplemented with 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International B.V.; Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotropin (hCG; Veterin Corion; Divasa Farmavic S.A.; Gurb, Barcelona, Spain). After 20-22 h, oocytes were transferred to 500 μL of pre-equilibrated maturation media without hormones.

Before fertilization, matured oocytes were denuded in Dulbecco's PBS (Gibco, ThermoFisher) and placed in 50-μL drops of pre-equilibrated *in vitro* fertilization medium with 1 mM of caffeine. The basic medium used for *in vitro* fertilization was a modified Tris-buffered medium (33). Semen samples were adjusted to a final concentration of 1,000 sperm per oocyte in fertilization medium.

Oocytes and sperm were co-incubated for 5 h. The presumptive zygotes were washed and transferred (40 zygotes/well) into a four-well multi-dish containing 500 μL of NCSU23 medium (34) supplemented with 0.4% BSA, 0.3 mM pyruvate and 4.5 mM lactate. After 2 days, cleaved embryos were counted

to calculate the fertilization rate; embryos were changed to NCSU23 medium supplemented with 0.4% BSA and 5.5 mM glucose, and cultured for 5 days. Embryos were classified following the Balaban & Gardner criterion (35) and the percentages of morulae, early blastocysts/blastocyst, hatching/hatched blastocysts and total embryos (sum of morulae, early blastocysts/blastocyst and hatching/hatched blastocysts) were calculated at Day 6 post-fertilization.

Oocyte maturation, *in vitro* fertilization and embryo culture were carried out at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

Statistical Analysis

Results were analyzed using a statistical package (IBM SPSS 25.0 for Windows; Armonk, NY, USA). Data were first check for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test).

The immunoblotting assay revealed two specific bands at ~80 kDa and 36 kDa. The ratio between 36 kDa and ~80 kDa, and the ratio between 36 kDa and the total intensity (corresponding to the sum of the two bands) were calculated to explore the relationship between the 36 kDa band and the different parameters analyzed.

The relative content of AKR1B1 in sperm was compared between ejaculated and epididymal samples through a Mann-Whitney test. In addition, ejaculates were classified into two groups based on the median of 36 kDa/~80 kDa and 36 kDa/total ratios. Sperm quality and functionality parameters, sperm resilience to preservation (resilience ratios) and *in vitro* fertilization outcomes were subsequently compared with a Mann-Whitney test. Correlations between sperm quality and functionality parameters, and 36 kDa/~80 kDa and 36 kDa/total ratios were calculated through Spearman coefficient. The level of significance was set at $P \leq 0.05$.

RESULTS

Identification and Quantification of AKR1B1 in Epididymal and Ejaculated Sperm

The first experiment aimed to evaluate whether AKR1B1 was present in epididymal and ejaculated sperm. Immunoblotting showed a double-band specific pattern for both samples at 36 kDa and ~80 kDa (**Figure 1A**). The specificity of the primary antibody was confirmed through incubating membranes with the AKR1B1 blocking peptide, as the two bands (36 kDa and ~80 kDa) disappeared (**Figure 1B**). Additionally, two pools of sperm lysates were subjected to urea denaturation to analyze whether the ~80 kDa disappeared, which would have indicated the dissociation of a potential AKR1B1 dimer. No changes, however, were observed in the band pattern between samples treated with and without urea (**Supplementary Figure 1**).

The quantification of the two bands in epididymal sperm resulted to be 0.16 ± 0.04 AU and 0.17 ± 0.05 AU for 36 kDa and ~80 kDa bands, respectively. In ejaculated sperm, the values were 0.12 ± 0.02 AU and 0.15 ± 0.04 AU for 36 kDa and ~80 kDa, respectively. No differences ($P > 0.05$) between epididymal and ejaculated sperm were found for any of the two bands (**Figure 1**).

Relationship Between Sperm AKR1B1 Levels and Sperm Quality Parameters After 0 and 72 h of Storage at 17°C

After confirming the presence of AKR1B1 in ejaculated sperm, the potential relationship between sperm AKR1B1 levels and sperm quality parameters (in terms of sperm morphology, motility and viability) in semen samples stored for 72 h at 17°C was evaluated. To determine the relationship between the 36 kDa band and these parameters, 36 kDa/~80 kDa and 36 kDa/total ratios were calculated and used for all the subsequent analysis. Sperm quality parameters were assessed at two time-points: immediately after ejaculate collection (0 h; sperm morphology, motility and viability) and after 72 h of preservation (sperm motility and viability). Spearman correlation coefficients between sperm quality parameters, assessed at both time-points, and 36 kDa/~80 kDa and 36 kDa/total ratios of sperm AKR1B1 were calculated (**Figure 2A**).

Regarding sperm morphology, the results revealed that 36 kDa/~80 kDa and 36 kDa/total ratios were positively correlated ($P < 0.05$) with the percentage of sperm with distal cytoplasmic droplets (R = 0.592 and R = 0.553, respectively). In addition, the percentages of viable sperm were positively correlated ($P < 0.05$) with both 36 kDa/~80 kDa and 36 kDa/total ratios at 0 h (R = 0.526 and R = 0.523, respectively), but not after 72 h of liquid storage ($P > 0.05$). While no relationship between 36 kDa/~80 kDa and 36 kDa/total ratios and total and progressive sperm motility was observed at 0 h ($P > 0.05$), a negative correlation ($P < 0.05$) between these two ratios and the percentages of total (R = -0.720 and R = -0.684, respectively) and progressively motile sperm (R = -0.610 and R = -0.576, respectively) assessed after 72 h of preservation was found. In addition, 36 kDa/~80 kDa and 36 kDa/total ratios were correlated ($P < 0.05$) to kinematic parameters evaluated at both time-points. Specifically, at 0 h, 36 kDa/~80 kDa and 36 kDa/total ratios were negatively correlated ($P < 0.05$) with VSL, VAP, LIN and WOB (VSL: R = -0.645 and R = -0.608; VAP: R = -0.696 and R = -0.673; LIN: R = -0.602 and R = -0.556; WOB: R = -0.702 and R = -0.686, respectively), and positively correlated ($P < 0.05$) with BCF (R = 0.605 and R = 0.601, respectively). After 72 h of storage, all kinematic parameters, except ALH and BCF, were negatively correlated ($P < 0.05$) with 36 kDa/~80 kDa and 36 kDa/total ratios (VCL: R = -0.571 and R = -0.554; VSL: R = -0.763 and R = -0.738; VAP: R = -0.678 and R = -0.666; LIN: R = -0.783 and R = -0.757; STR: R = -0.723 and R = -0.680; WOB: R = -0.705 and R = -0.700, respectively).

Semen samples were classified into two groups according to their 36 kDa/~80 kDa and 36 kDa/total ratios of sperm AKR1B1, with high (2.3 ± 0.092 AU and 0.7 ± 0.01 AU, respectively [n = 8]) or low 36 kDa levels (1.5 ± 0.46 AU and 0.6 ± 0.08 AU, respectively [n = 8]). Then, sperm quality parameters were compared between the two groups (**Figure 2B**). In the case of sperm morphology, only the

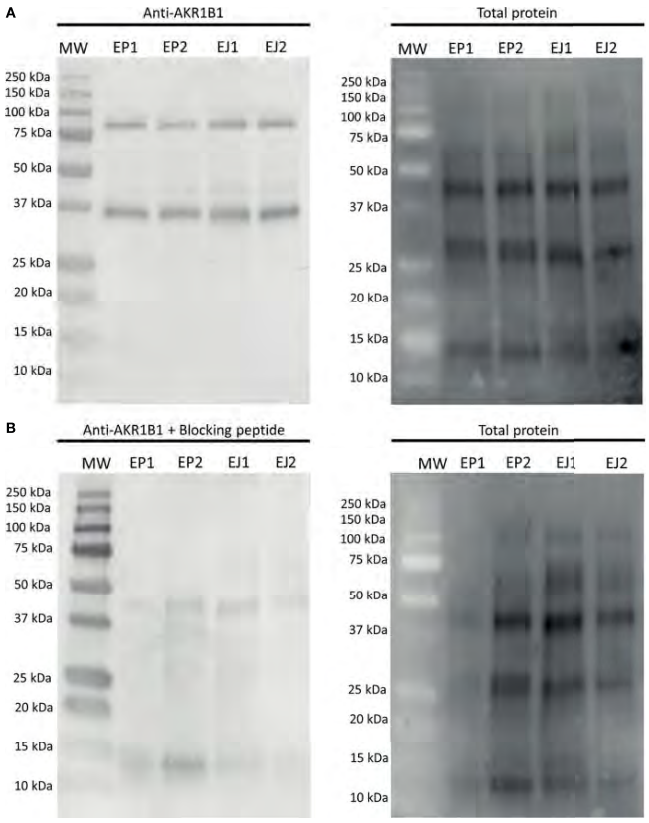

FIGURE 1 | Representative Western blot analysis of **(A)** anti-AKR1B1 and **(B)** the corresponding blocking peptide in epididymal and ejaculated sperm, and their total protein controls for both membranes. MW, molecular weight; EP1 and EP2, epididymal sperm samples; EJ3 and EJ4, ejaculated sperm samples.

percentage of sperm with distal droplets differed between groups ($P < 0.05$), being greater in the high than in the low 36 kDa levels group (3.1 ± 1.74% *vs.* 1.2 ± 1.42%, respectively). In addition, sperm viability only differed ($P < 0.05$) at 0 h, being greater in the group with high than in that with low 36 kDa levels (92.2 ± 2.12% *vs.* 88.4 ± 3.91%, respectively). As far as sperm motility is concerned, the percentages of total and progressively motile sperm after 72 h of liquid storage were larger in the low than in the high 36 kDa levels group (total sperm motility: 61.3% ± 25.97% *vs.* 24.4% ± 21.93%; progressive sperm motility: 48.5% ± 32.49% *vs.* 10.1% ± 11.45%, respectively). Moreover, VSL, VAP, LIN and WOB were significantly higher ($P < 0.05$) in the group with low than in that with high levels of 36 kDa at both evaluation time-points (VSL: 36.9 ± 13.02% *vs.* 24.7 ± 8.02% at 0 h and 34.2 ± 15.51% *vs.* 13.9 ± 8.98% at 72 h; VAP: 43.8 ± 13.49% *vs.* 31.2 ± 7.02% at 0 h and 38.2 ± 14.88% *vs.* 21.5 ± 12.74% at 72 h; LIN: 60.0 ± 15.63% *vs.* 42.5 ± 16.22% at 0 h and 67.8 ± 13.68% *vs.* 38.2 ± 14.13% at 72 h; WOB: 70.9 ± 13.64% *vs.* 53.08 ± 13.03% at 0 h and 77.5 ± 8.02% *vs.* 59.4 ± 13.15% at 72 h, respectively). On the other hand, STR and BCF only differed ($P < 0.05$) between groups after 72 h of preservation, displaying higher values in the low than in the high 36 kDa levels group (STR: 86.7 ± 11.08% *vs.* 62.5 ± 11.36%; BCF: 8.1 ± 0.70% *vs.* 5.6 ± 2.86%, respectively).

Relationship Between Sperm AKR1B1 Levels and Sperm Functionality Parameters After 0 h and 72 h of Preservation

The relationship between sperm AKR1B1 (36 kDa/~80 kDa and 36 kDa/total ratios) and sperm functionality parameters (acrosome integrity, mitochondrial membrane potential and intracellular calcium levels) after 0 h and 72 h of storage at 17°C was also investigated through Spearman correlation (**Figure 3A**).

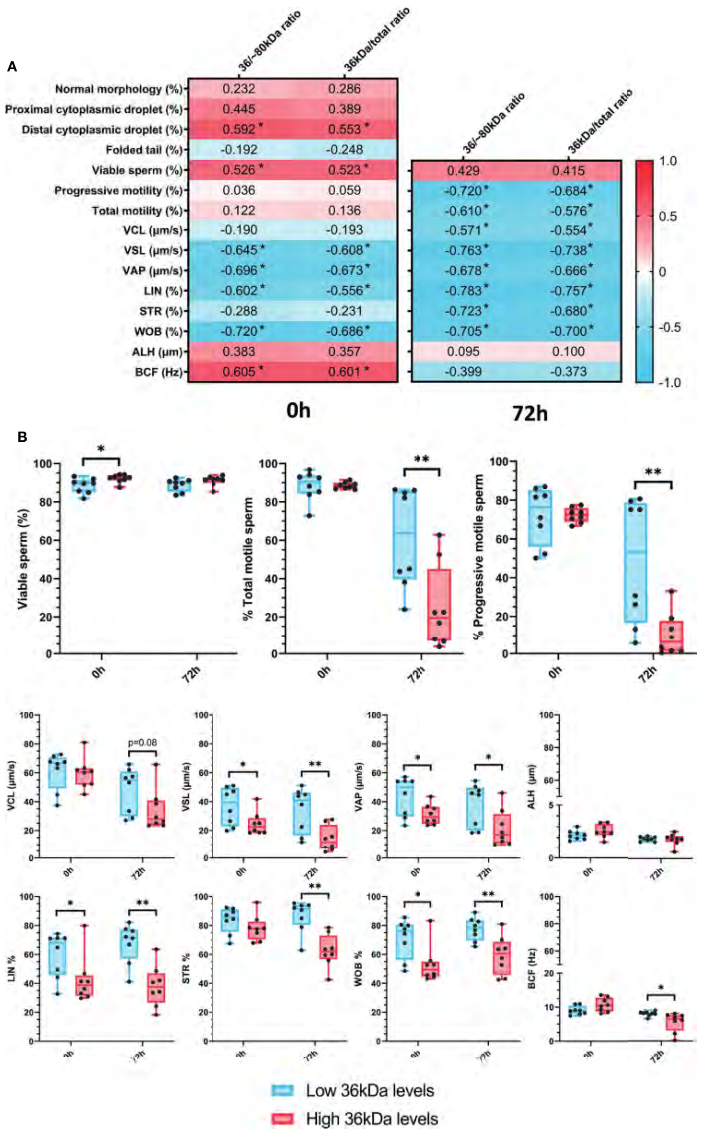

FIGURE 2 | **(A)** Correlation plot of sperm quality parameters (including sperm morphology, motility and viability) and 36/~80 kDa and 36 kDa/total ratios. Semen samples of 16 AI-boars (one ejaculate per boar) were evaluated immediately after semen samples arrived at the laboratory (0 h) and after storage/preservation at 17°C for 72 h. The color saturation of red to blue represents the correlation coefficients (R) from 1 to -1, respectively. Significant correlations ($P < 0.05$) are marked with *. **(B)** Differences between groups with high and low levels of the 36 kDa band for viability and motility parameters evaluated at both 0 h and 72 h The box indicates the maximum and the minimum of each group, and the thicker line the median. Each dot represents one semen sample. Significant differences ($P < 0.05$) are marked with * and ($P < 0.01$) are marked with **.

A negative correlation ($P < 0.05$) between intracellular calcium levels at both evaluation time-points and the two ratios was found (0 h: R = -0.842 and R = -0.832; and 72 h: R = -0.651 and R = -0.574, respectively). In contrast, no correlation between 36 kDa/~80 kDa and 36 kDa/total ratios and the other sperm functionality parameters was observed ($P > 0.05$).

Sperm functionality parameters were also compared between the two groups (with high or low 36 kDa levels; **Figure 3B**). Whereas intracellular calcium levels were significantly ($P < 0.05$) greater in the low than in the high 36 kDa levels group after both 0 h and 72 h of preservation (0 h: 32,716.0 ± 21,328.35 AU *vs.* 6,616.0 ± 14,258.50 AU; 72 h: 26,204.9 ± 17,784.10 AU *vs.* 5,581.8 ± 94.25 AU, respectively), no significant differences in the other variables were found.

Relationship Between Sperm AKR1B1 Levels and the Sperm Ability to Withstand Refrigeration for 72h

The present report also aimed to evaluate whether sperm AKR1B1 could be related to the sperm resilience to

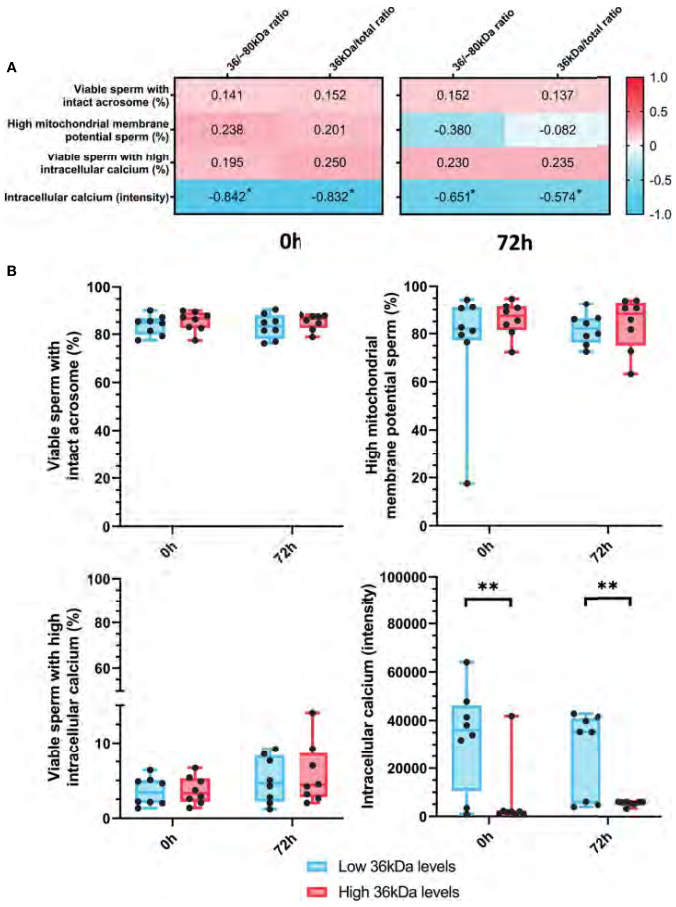

FIGURE 3 | **(A)** Correlation plot of sperm functionality parameters (including acrosome integrity, mitochondrial membrane potential and intracellular calcium) and 36/~80 kDa and 36 kDa/total ratios. Semen samples of 16 AI-boars (one ejaculate per boar) were evaluated immediately after semen samples arrived at the laboratory (0 h) and after storage/preservation at 17°C for 72 h. The color saturation of red to blue represents the correlation coefficients (R) from 1 to -1, respectively. Significant correlations ($P < 0.05$) are marked with *. **(B)** Differences between groups with high and low levels of the 36 kDa band for the functionality parameters evaluated at both 0 h and 72 h. The box indicates the maximum and the minimum of each group and the thicker line the median. Each dot represents one semen sample. Significant differences ($P < 0.01$) are marked with **.

preservation, as this could also be considered as an indicator of semen quality. To this end, quotients between the values of each parameter at 72 h and 0 h were calculated and defined as resilience ratios (e.g. % Progressive motility after 72 of preservation/% Progressive motility at 0 h; **Figure 4**). Regarding sperm quality parameters, Spearman correlation analysis showed that 36 kDa/~80 kDa and 36 kDa/total ratios were negatively correlated ($P < 0.05$) with resilience ratios for progressive motility (R = -0.807 and R = -0.775, respectively), total motility (R = -0.627 and R = -0.592, respectively), VSL (R = -0.712 and R = -0.708, respectively), STR (R = -0.725 and R = -0.713, respectively) and BCF (R = -0.654 and R = -0.634, respectively). Concerning sperm functionality parameters, 36 kDa/~80 kDa and 36 kDa/total ratios were positively correlated ($P < 0.05$) with resilience ratios for viable sperm with high intracellular calcium levels (R = 0.643 and R = 0.616, respectively). No other correlations were found.

In addition, resilience ratios were also compared between the two groups with high and low 36 kDa levels. Resilience ratios for progressive and total motility, VSL, STR and BCF were greater in the low than in the high 36 kDa levels group (total sperm motility: 68.6 ± 26.03% *vs.* 27.8 ± 25.12%; progressive sperm motility: 63.7 ± 35.40% *vs.* 13.7 ± 15.04%; VSL: 0.89 ± 0.185 *vs.* 0.53 ± 0.233; STR: 1.04 ± 0.082 *vs.* 0.80 ± 0.135; BCF: 0.91 ± 0.150 *vs.* 0.52 ± 0.278, respectively). Furthermore, and due to the different distribution of samples between groups, the resilience ratio for sperm viability was also greater in the low than in the high 36 kDa levels group ($P < 0.05$; 1.00 ± 0.015% *vs.* 0.99 ± 0.009%, respectively). No differences between groups were found for the other sperm quality and functionality parameters ($P > 0.05$).

Relationship Between Sperm AKR1B1 Levels and *In Vitro* Fertilizing Ability

The relationship between sperm AKR1B1 levels and *in vitro* fertility outcomes was also explored in the present work. Spearman correlation coefficients between 36 kDa/~80 kDa and 36 kDa/total ratios and *in vitro* fertility parameters (fertilization rate at day 2; percentages of total embryos, morulae, early blastocysts/blastocysts and hatching/hatched blastocysts at day 6) are depicted in **Figure 5A**. Fertilization rate at day 2 negatively correlated ($P < 0.05$) with both 36 kDa/~80 kDa and 36 kDa/total ratios (R = -0.424 and R = -0.451, respectively). Similarly, a negative correlation ($P < 0.05$) between the percentage of total embryos at day 6 and the two ratios was found (R = -0.531 and R = -0.495, respectively).

In vitro fertility parameters were also compared between the two groups (high or low 36 kDa band levels; **Figure 5B**). Greater fertilization rate at day 2 ($P < 0.05$) was found in the group with low than in that with high 36 kDa levels (38.8 ± 12.34% *vs.* 29.7 ± 7.12%, respectively). Similarly, the percentage of total embryos at day 6 was significantly greater ($P < 0.05$) in the low than in the high 36 kDa levels group (51.4 ± 12.63% *vs.* 38.4 ± 8.09%, respectively). No differences between groups in any of the other IVF outcomes were found ($P > 0.05$).

DISCUSSION

Aldose Reductase B1 in SP has been proposed as a potential *in vivo* fertility marker (8), but not as a sperm quality and functionality predictor (21). In spite of this, little information regarding the role of this protein when present in sperm exists in the literature. For this reason, the present work aimed to investigate the relationship of sperm AKR1B1 with sperm quality, function and fertilizing ability using the pig as an animal model. The results of the present work showed, for the first time in mammalian species, that: i) the relative content of AKR1B1 does not differ between ejaculated and epididymal sperm; ii) the levels of the 36 kDa band detected after AKR1B1 immunoblotting are related to sperm motility and kinematic parameters; iii) sperm having greater content of the 36 kDa band show lower intracellular calcium levels; iv) the levels of the 36 kDa band are related to the sperm resilience to liquid preservation; and v) sperm with smaller content in the 36 kDa

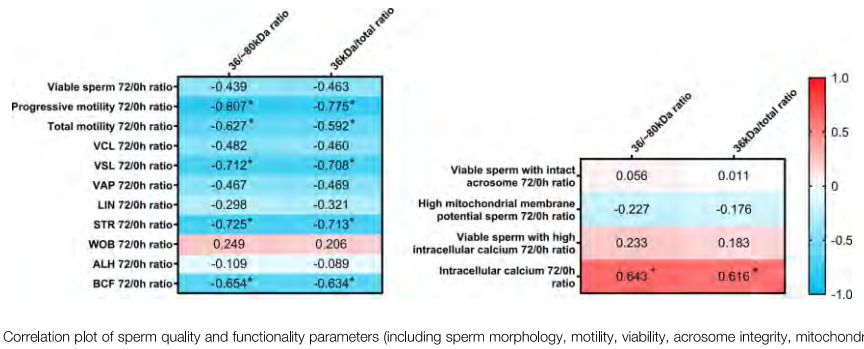

FIGURE 4 | Correlation plot of sperm quality and functionality parameters (including sperm morphology, motility, viability, acrosome integrity, mitochondrial membrane potential and intracellular calcium) 72/0 h ratios and 36/~80 kDa and 36 kDa/total ratios. Semen samples of 16 AI-boars (one ejaculate per boar) were evaluated immediately after semen samples arrived at the laboratory (0 h) and after storage/preservation at 17°C for 72 h, and the ratio72/0h was calculated for each. The color saturation of red to blue represents the correlation coefficients (R) from 1 to -1, respectively. Significant correlations ($P < 0.05$) are marked with *.

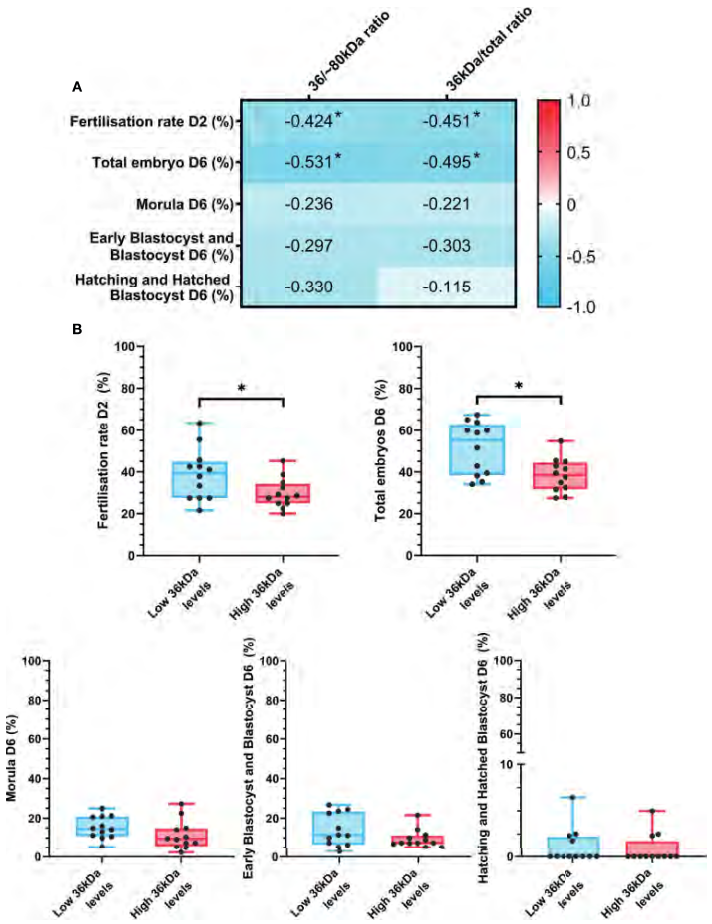

FIGURE 5 | **(A)** Correlation plot of *in vitro* fertility outcomes (evaluated as fertilization rate at day 2 and percentages of total embryos, morulas, early blastocysts/blastocyst and hatching/hatched blastocysts at day 6) and 36/~80 kDa and 36 kDa/total ratios. *In vitro* fertility procedure was performed using samples from 24 AI-boars (one ejaculate per boar). The color saturation of red to blue represents the correlation coefficients (R) from 1 to -1, respectively. Significant correlations ($P < 0.05$) are marked with *. **(B)** Differences between groups with high and low levels of the 36 kDa band for *in vitro* fertility parameters. The box indicates the maximum and the minimum of each group and the thicker line the median. Each dot represents one semen sample. Significant differences ($P < 0.05$) are marked with *.

band lead to higher fertilization rate at day 2 and percentage of total embryos at day 6 post-fertilization.

The presence or activity of AKR1B1 in ejaculated sperm has been demonstrated in equine (26), bovine (17, 25) and porcine species (24). Similarly, the results of the peptide competition assay of the Western Blot showed a specific double-band pattern at 36 kDa and ~80 kDa in all ejaculated and epididymal sperm samples, thus suggesting that different forms of this protein could be physiologically present in pig sperm. In effect, this same pattern has been recently observed in the boar reproductive tract, specifically in the testis, epididymis, prostate and seminal vesicles (21), in the ovine thymus and spleen (19, 36) and in bovine peripheral blood mononuclear cells (37). After confirming that the epitope detected by the antibody was specific for AKR1B1 and not for other similar proteins, we suspected that the ~80 kDa band could correspond to a dimeric form of AKR1B1. The dimerization of both xylose reductase and AKR7, also members of the AKR superfamily, have been associated to the active form of this protein (38–41). An AKR1B1 dimer (hypothetically, the ~80 kDa band),

therefore, could reasonably be assumed to be the active form of this protein, as already proposed by other authors (37). Yet, our additional experiment assessing the denaturation of the putative dimeric protein extract through urea did not confirm this hypothesis. Thus, it cannot be discarded that the ~80 kDa band corresponds to the covalent union of AKR1B1 with other proteins. In spite of this, considering that both bands are specific, our analysis of the relationship between this protein and fertility outcomes envisaged 36/~80 kDa and 36 kDa/total ratios as a measurement of the possible activation state of AKR1B1.

Aldose reductase has been reported to be transferred to sperm during epididymal maturation, as its content increases along the epididymal transit (18, 24). Yet, to the best of our knowledge, no study has addressed whether AKR1B1 levels in sperm are increased upon ejaculation due to the acquisition of this protein from the extracellular vesicles present in SP (e.g. prostasomes). The results of the immunoblotting analysis of the present work did not show differences in the relative AKR1B1 content between ejaculated and epididymal sperm, thus suggesting that the AKR1B1 contained in ejaculated sperm is mainly acquired during sperm maturation rather than at ejaculation. Remarkably, aldose reductase activity has been widely associated to epididymal maturation in bovine, murine and human species (17, 22–24). Specifically, AKR1B1 has been suggested to modulate: i) bovine and murine sperm motility through the polyol pathway (23, 25) and, ii) bovine sperm survival (25) during epididymal maturation. Considering the previous results and the existing literature, the present work evaluated if AKR1B1 levels are related to different secondary morphological abnormalities arising from an epididymal origin (27). In this regard, sperm AKR1B1 was found to be related to the percentages of sperm with distal cytoplasmic droplets, which were found to be greater when the levels of the 36 kDa band (putative inactive form of AKR1B1) were higher. It is worth mentioning that the strong genetic selection of AI-boars during the last decades has left only highly fertile individuals and, for this reason, morphological abnormalities related to inefficient epididymal maturation may not be noticeable in studies conducted in this species. The relationship between sperm AKR1B1 and the presence of distal cytoplasmic droplets, nevertheless, is in agreement with the literature (27), as higher levels of the inactive AKR1B1 form would contribute to increase the presence of morphological abnormalities originated during sperm maturation.

Besides its liaison with epididymal maturation, AKR1B1 has been found to influence ejaculated sperm physiology (24). For this reason, the present work also aimed to explore the potential relationship between the AKR1B1 and sperm functionality and *in vitro* fertilization outcomes. One of the main results of this work was that higher levels of the 36 kDa band were strongly related to lower intracellular calcium levels. Intracellular calcium is known to modulate multiple signaling pathways, the one regulating sperm motility being very relevant. In effect, increases in intracellular calcium levels are required for mammalian sperm to switch to hyperactive movement (42). Interestingly, the present work also observed a clear influence of AKR1B1 levels on several kinematic parameters. For this reason, it could be hypothesized that the participation of AKR1B1 in the regulation of intracellular calcium levels could ultimately affect sperm motility. This would be in agreement with previous studies in which AKR1B1 was found to modulate sperm motility during epididymal maturation in cattle (25) and mice (23), and during sperm capacitation in pigs (24). Remarkably, in this last study carried out in pigs, the authors found that aldose reductase was able to regulate the change from progressive to hyperactivated movement during capacitation (24). While the present work did not investigate the involvement of AKR1B1 in sperm capacitation, no relationship between acrosome integrity and AKR1B1 levels was found. For this reason, and due to the strong relationship found with intracellular calcium, which may have implications in sperm capacitation, further research is needed to clarify the precise implication of AKR1B1 in this process.

Liquid storage is widely used to preserve mammalian sperm up to 3-5 days (43). Yet, during this process there is a gradual decline of sperm quality and functionality (44, 45) and, for this reason, sperm resilience to preservation can be considered as a good semen quality and functionality indicator. Recently, our research group evaluated the potential relationship between AKR1B1 levels in pig SP and sperm quality and functionality parameters assessed after 72 h of liquid stored at 17°C, showing that the levels of this protein were not related to these parameters (21). On the contrary, the present study found that 36/~80 kDa and 36 kDa/total ratios in pig sperm are related to the sperm ability to withstand liquid storage for 72 h. Briefly, the results showed that higher levels of the potentially inactive AKR1B1 form negatively influenced the preservation of sperm motility (in terms of progressive and total motility and several kinematic parameters). These results suggest that sperm AKR1B1 might be involved in the resilience to cellular stress, evaluated here as sperm liquid storage. Although, to the best of our knowledge, no study has addressed the potential role of AKR1B1 in coping sperm stress, aldose reductases present in cattle embryos have been found to be upregulated against heat stress (46, 47). The positive effect in the resilience to this stress could be driven by the antioxidant activity of AKR1B1 (12), because this protein has already been reported to modulate ROS production at least during pig sperm capacitation (24). This hypothesis, nonetheless, should be addressed in future studies.

Considering that AKR1B1 from SP has been found to affect *in vivo* fertility outcomes (8), the present work also aimed to determine whether sperm AKR1B1 is related to *in vitro* fertilizing ability. The results showed that increased levels of the 36 kDa band (putative inactive form of AKR1B1) were negatively related to fertilization rate at day 2 and the percentage of total embryos at day 6 post-fertilization. As mentioned before, aldose reductase has already been proposed to be an essential factor for sperm function, because it modulates sperm capacitation and this could affect their fertilizing competence (24). This is supported by the findings of this work, as sperm AKR1B1 has been found to play an active role in the regulation of sperm motility. As mechanical penetration of zona pellucida is facilitated through sperm motility hyperactivation (48, 49), lower levels of the active

protein could hinder oocyte penetration and, thus, fertilization. Indeed, as confirmed in the present study by day 6 observations, this lower fertilization rate is likely to result in a lower percentage of embryos. For all these reasons, it is reasonable to assume that high levels of the sperm active AKR1B1 form underlie an increased sperm fertilizing potential and, consequently, there is a positive relationship with fertility outcomes.

To conclude, aldose reductase has been widely reported to be essential for both female and male reproductive physiology. Focusing on the male, the results presented in this work showed that sperm AKR1B1 is related to epididymal maturation and modulation of sperm motility, probably through signaling pathways involving calcium homeostasis. Moreover, sperm AKR1B1 seems to have an effect on the sperm ability to withstand stress, measured in the present work as resilience to preservation. Finally, sperm AKR1B1 has also been reported to affect *in vitro* fertility outcomes, possibly through the modulation of sperm fertilizing potential. Further studies, nevertheless, are required to elucidate how AKR1B1 influences cellular stress, sperm capacitation and fertilization. Bearing in mind the current knowledge on aldose reductases, several hypotheses could be raised. First, considering that AKR1B1 can act as a detoxifying enzyme (14, 23, 39), it could exert its effect on sperm physiology through the regulation of intracellular ROS levels. Alternatively, because aldose reductase is postulated as a crucial enzyme in the polyol pathway (17, 22, 23), understanding the function of AKR1B1 in pig sperm metabolism could also help understand the relevance of that pathway in ejaculated sperm physiology and fertilizing capacity. Finally, it is likely that a balanced combination of both mechanisms promotes an optimal sperm function, thus positively influencing sperm fertilizing ability.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

No animal was manipulated by the authors, as ejaculated semen samples were acquired from a local farm (AI-center) and the abattoir donated the epididymis of boars that were sacrificed for genetic replacement reasons. Therefore, no permission from an Ethics Committee was required.

AUTHOR CONTRIBUTIONS

Conceptualization: YM-O, JR-M, and MY. Methodology: YM-O, ML, AD-B, SR, and JR-M. Formal analysis and investigation: YM-O and MY. Writing - original draft preparation: YM-O. Writing - review and editing: IB, MY, and JR-M. Funding acquisition: MY. Supervision: JR-M, IB, and MY. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.773249/full#supplementary-material

Supplementary Figure 1 | Western blot analysis (anti-AKR1B1 antibody) following 16 M urea denaturation of sperm samples. The control consisted of sperm samples that were not incubated with 16 M urea. MW, molecular weight; S1 and S2, sperm lysate samples; S1U and S2U, sperm lysate samples incubated with 16 M urea.

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Paper V

Sperm physiology and *in vitro* fertility outcomes rely on their basal metabolic activity: insights from the pig model.

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Sperm physiology and in vitro fertilising ability rely on basal metabolic activity: insights from the pig model

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Whether basal metabolic activity in sperm has any influence on their fertilising capacity has not been explored. Using the pig as a model, the present study investigated the relationship of energetic metabolism with sperm quality and function (assessed through computer-assisted sperm analysis and flow cytometry), and fertility (in vitro fertilisation (IVF) outcomes). In semen samples from 16 boars, levels of metabolites related to glycolysis, ketogenesis and Krebs cycle were determined through a targeted metabolomics approach using liquid chromatography-tandem mass spectrometry. High-quality sperm are associated to greater levels of glycolysis-derived metabolites, and oocyte fertilisation and embryo development are conditioned by the sperm metabolic status. Interestingly, glycolysis appears to be the preferred catabolic pathway of the sperm giving rise to greater percentages of embryos at day 6. In conclusion, this study shows that the basal metabolic activity of sperm influences their function, even beyond fertilisation.

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The spermatozoon has historically been regarded as a mere vehicle to deliver the paternal genome into the oocyte; consequently, the importance of paternal factors for oocyte fertilisation, embryo development and, even, offspring health, has been traditionally overlooked. This has led to the misconception that the male contribution to early embryo development solely relies on the sperm genome in terms of DNA integrity and epigenetic signatures[1]. In the last decades, however, multiple studies demonstrated that sperm proteome (reviewed in ref. [2]), lipidome (reviewed in ref. [3]) and transcriptome (reviewed in ref. [4]) also have a crucial influence on oocyte fertilisation and embryo development in mammals. Despite the fact that the sperm metabolome has been proven to affect male fertility in several mammalian species (reviewed in ref. [5]), the repercussion of sperm bioenergetics on oocyte fertilisation and embryo development remains unknown.

Mammalian sperm consume ATP for a wide range of functions, including capacitation, hyperactivation, acrosome reaction and oocyte penetration, each occurring in different environments. Upon ejaculation, sperm come into contact with seminal plasma (SP) and, when deposited within the female reproductive tract, they interact with uterine and oviductal fluids, which are known to differ in terms of ion[6] and metabolite[7–9] composition. Changes in these surrounding biofluids and, therefore, the availability of substrates and oxygen, together with the dramatic changes that sperm undergo to become fertilising competent[10], force these cells to utilise diverse metabolic pathways, including glycolysis and oxidative phosphorylation (Oxphos)[11,12] to satisfy their energetic requirements. In addition, the metabolic pathway preferentially used by sperm is highly species-specific[12]. While glycolysis appears to be the main energetic pathway in humans and rodents[13,14], Oxphos seems to be predominant in horses[15], and a balance of both occurs in cattle[16]. There is, notwithstanding, a discrepancy in the metabolic pathway preferred by pig sperm[13,17]. Remarkably, the diversity of conditions to which sperm are subject to during their life cycle, along with the species-specific differences in their metabolism, contribute to the ongoing debate surrounding the catabolic pathways used by these cells[18].

Because, in sperm, mitochondrial membrane potential, an indirect method to estimate energy production, has been found to affect in vitro fertilisation (IVF) outcomes[19–22], it is reasonable to hypothesise that energetic-related metabolites, e.g., metabolites linked to glycolysis, ketogenesis and Oxphos, are also involved. In this sense, targeted metabolomics approaches offer the possibility of exploring cellular metabolism and metabolic status under specific conditions. Thus, by identifying metabolites in biological samples such as fluids or cell extracts, one can describe physiological processes, generate new hypotheses for unsolved metabolic interrogations and even find potential biomarkers[23]. Against this background, the present study aimed to address: (1) the metabolic pathway preferred by porcine sperm to produce energy; 92) whether the energetic metabolic state of sperm is related to their quality and function; and (3) the potential relationship between sperm energetic metabolism and oocyte fertilisation and subsequent embryo development. In addition, the pig has been proposed as a suitable animal model, not only on the grounds of the physiological similarities with humans and the availability of semen samples of high volume but also because rodent species may not be appropriate for sperm physiology studies, as epididymal sperm are never in contact with SP[24]. Yet, before their use for metabolomic studies, the energetic pathway utilised by pig sperm and how similar this is to their human counterparts needs to be elucidated. To this end, sperm samples were split into three aliquots. Two were used to evaluate sperm quality/function parameters and run IVF experiments, respectively. The other was intended to quantify sperm intracellular metabolites related to glycolysis, ketogenesis, polycarboxylic acids cycle and Oxphos through liquid chromatography-tandem mass spectrometry (LC-MS/MS), which provides highly specific, sensitive, accurate and reproducible results[25].

Results

Dimensionality reduction. Three different blocks were included to assess sperm physiology and in vitro fertility: sperm quality, sperm function and IVF outcomes. Parameters included in the sperm quality block were the percentage of sperm with normal morphology, the percentage of motile sperm (total motility), the percentage of sperm with progressive motility (progressive motility) and the percentage of viable sperm. The first principal component (PC) from principal component analysis (PCA) of this block represented up to 77% of total variability. The sperm function block was comprised of three different variables: the percentage of viable sperm with an intact acrosome, intracellular calcium levels and the percentage of sperm with high mitochondrial membrane potential; one outlier was detected and excluded from this block. In this case, the first PC explained 46% of the total variability. The IVF outcomes block encompassed fertilisation rate at day 2 post-fertilisation and the different embryo developmental stages evaluated 6 days after fertilisation, which included: percentages of morulae, early blastocysts/blastocysts and hatched/hatching blastocysts; the sum of morulae, early blastocysts/blastocysts and hatched/hatching blastocysts; and the total number of embryos. Two additional ratios were also calculated: the developmental potential of embryos at day 6 and the developmental competency of fertilised embryos. The PCA of this dataset showed 50% of the total variability in the first PC. Additional information is provided in Supplementary Table 1.

Association of metabolic signature with sperm physiology and IVF outcomes. The Partial Least Squares (PLS) model denoted an association between metabolites and sperm physiology and in vitro fertility reduced dimension feature vectors. The performance of the different models showed a cross-validated R^2 of 0.823, 0.830 and 0.460 for sperm quality, sperm function and IVF outcomes, respectively. Likewise, predictive Q_2 values of 0.269, 0.693 and 0.387 were obtained for the aforementioned blocks (Fig. 1). The predictive capability of these models was validated with a permutation test (P value of 0.013, <0.001 and <0.001 for sperm quality, sperm function and IVF outcomes, respectively; Fig. 1). The included feature selection of the model exhibited several metabolites associated to each block, as shown in Table 1. Overall, citrate, isocitrate, lactate, citrate/lactate and citrate/malate were found to be linked to sperm quality, sperm function and IVF outcomes. In addition, α-hydroxyglutarate/citrate, α-hydroxyglutarate/isocitrate and α-ketoglutarate/isocitrate were also related to both sperm quality and sperm function. Finally, acetoacetate, fumarate and isocitrate/citrate were found to be correlated with sperm quality.

Multi-block data analysis reveals specific metabolic processes associated with sperm physiology and IVF variables. Once metabolic markers associated with sperm physiology and IVF inputs were detected, whether these observations were intercorrelated across the dataset was investigated. For this purpose, the relationships between features observed from the N-integration with Projection to Latent Structures model across data were examined and visualised in an integrative network, as shown in Fig. 2. For subsequent analyses, the sample containing the previously detected outlier in the PCA of the sperm function block was excluded. The correlation of latent components between blocks is summarised in Supplementary Table 2.

Multi-block data analysis revealed that not only were sperm physiology and in vitro fertility blocks closely related to the metabolomics one but also with each other. Unfolded pair-wise

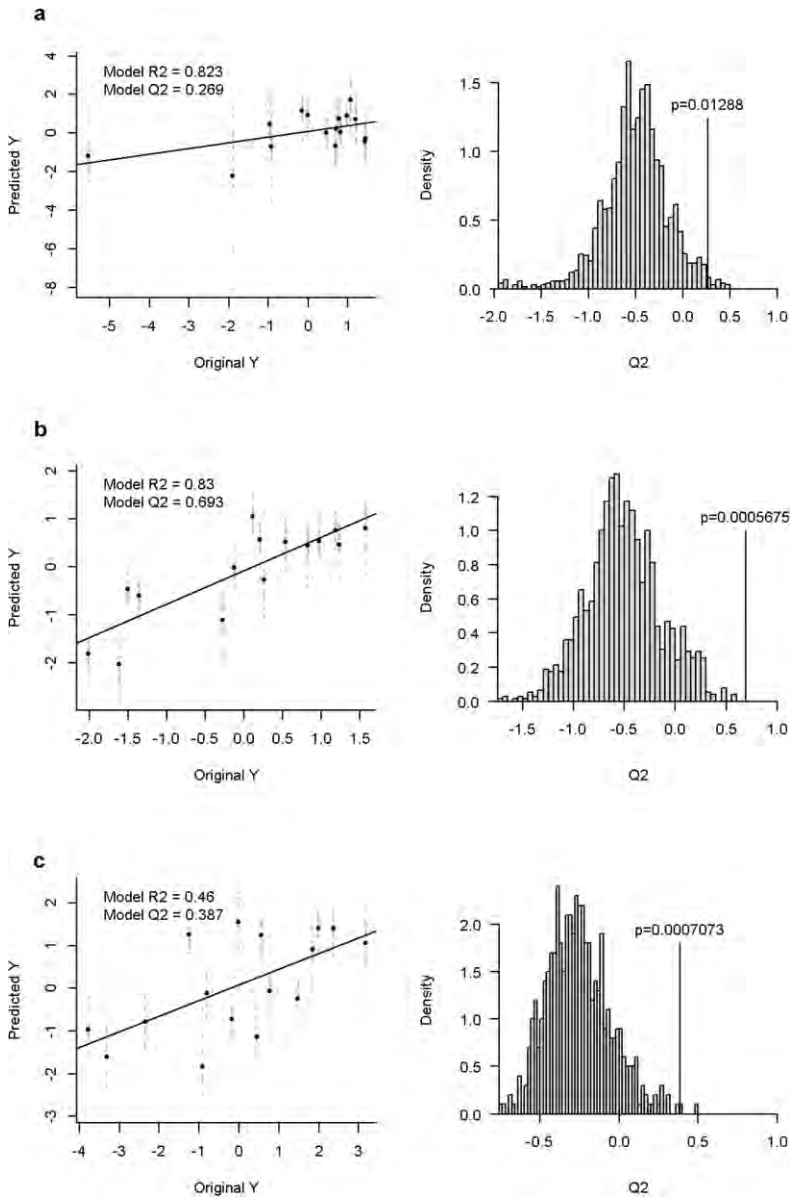

Fig. 1 Partial least square (PLS) regression plots of actual and predicted. a sperm quality ($n = 16$), **b** sperm function ($n = 15$) and (**c**) in vitro fertilisation outcomes ($n = 16$). Reduced-dimension feature vectors from projection are shown in the left, and permutation tests based on the prediction capability are depicted on the right. In the x-axis, PLS regression plots show original centred, reduced-dimension feature vectors from each principal component analysis. The values predicted by the models are displayed on the *y* axis. The slope is defined by the prediction capability values (Q2) tested through permutation tests. The vertical lines of permutation test plots indicate the Q2 values obtained in each block.

Table 1 Metabolites associated with sperm physiology and in vitro fertility in the PLS models.

	Metabolites	Recursive rank[a]	LR[b]	*P* value[c]	FDR	Beta[c]
Sperm quality	Acetoacetate	3.64	1	<0.001	0.002	0.04
	Lactate	5.52	2	0.006	0.035	<0.01
	Citrate	7.73	3	0.991	0.991	<0.01
	Citrate/malate	8.26	4	0.343	0.573	<0.01
	Fumarate	8.37	5	0.008	0.036	0.01
	α-hydroxyglutarate/isocitrate	9.81	6	0.781	0.879	<0.01
	Isocitrate	9.81	7	0.056	0.126	0.01
	Citrate/lactate	9.96	8	0.350	0.573	<0.01
	α-Ketoglutarate/Isocitrate	12.76	9	0.529	0.793	<0.01
	Isocitrate/citrate	-	-	<0.001	0.002	9.77
Sperm function	Citrate	2.03	1	<0.001	0.002	−1.27
	Citrate/lactate	2.05	2	<0.001	0.001	−1.35
	Citrate/malate	3.82	3	<0.001	0.005	−1.27
	α-hydroxyglutarate/isocitrate	6.53	4	0.005	0.018	1.25
	α-Ketoglutarate/isocitrate	-	-	0.005	0.018	1.40
	Isocitrate	-	-	0.019	0.058	−1.39
IVF outcomes	Citrate/lactate	3.37	1	<0.001	0.003	−0.32
	Citrate/malate	3.41	2	0.003	0.029	−0.42
	Citrate	4.67	3	0.007	0.041	−0.37
	α-Ketoglutarate	9.71	4	0.092	0.251	−0.42
	Isocitrate	10.25	5	0.050	0.181	−0.26
	Lactate	-	-	0.044	0.181	0.29

FDR false discovery rate.
[a]Recursive rank of double-cross-validation PLS regression. In each block, metabolites were repeatedly ranked in each outer iteration and cumulated in the recursive rank feature. Only significant variables are numbered.
[b]Loading rank displays the absolute ranking of variables based on their importance.
[c]*P* and beta values of linear models. *P* values were corrected utilising the Benjamini–Hochberg formula.

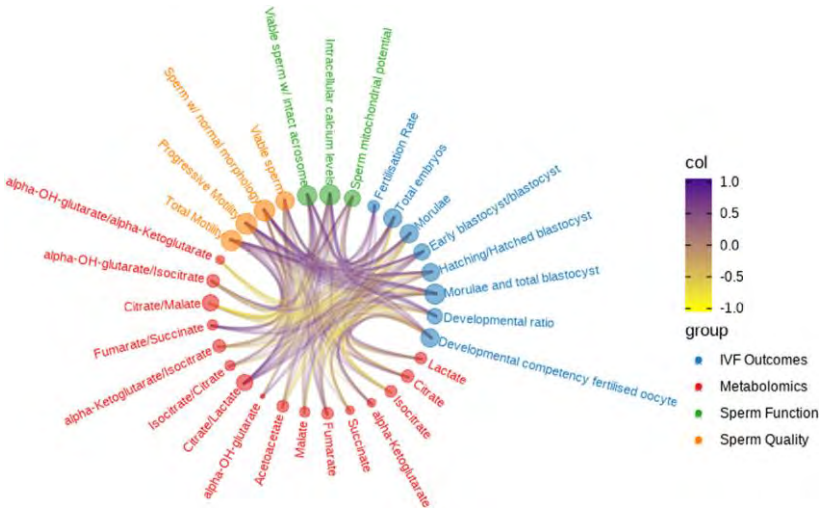

Fig. 2 Integrative network graph depicting correlations derived from the N-integration with projection to latent structures between blocks. In vitro fertilisation outcomes (blue), metabolomics (red), sperm function (green) and sperm quality (orange) were associated on the basis of a similarity score > | 0.3| ($n = 15$). Lines are coloured according to similarity scores: positive associations are shown in purple, whereas inverse associations are depicted in yellow. Nodes (circles) represent variables and are sized according to the number of connections. Further information can be found in Supplementary Table 3. col colour, IVF in vitro fertilisation, OH hydroxy.

similarity scores from multi-block data integration are shown in Supplementary Table 3. The general trend was that whereas citrate, isocitrate and citrate/malate showed a positive association with sperm quality and in vitro fertility blocks, α-hydroxyglutarate/isocitrate, α-hydroxyglutarate/α-ketoglutarate and α-ketoglutarate/citrate exhibited a negative relationship. Interestingly, these associations were found to be inverse in the case of sperm function parameters.

Focusing on sperm quality, the most relevant positive relationships were noted between (i) the percentage of sperm with progressive motility and citrate, citrate/lactate and citrate/malate (similarity score >0.70); (ii) the percentage of sperm with normal morphology and citrate, citrate/lactate and citrate/malate (similarity score >0.70); and (iii) the percentage of viable sperm and citrate, citrate/lactate and citrate/malate (similarity score >0.50). On the other hand, scarce negative relationships were observed between sperm quality parameters and metabolites. The most relevant interactions were found between (i) the percentage of sperm with progressive motility and α-ketoglutarate/isocitrate and α-hydroxyglutarate/isocitrate (similarity score = −0.67 and −0.58, respectively); and (ii) the percentage of sperm with normal morphology and α-hydroxyglutarate/isocitrate (similarity score = −0.61).

Regarding sperm function, a strong positive relationship (similarity score >0.70) was seen between (i) the percentage of sperm with high mitochondrial membrane potential and α-hydroxyglutarate/α-ketoglutarate; (ii) intracellular calcium levels and α-hydroxyglutarate/isocitrate and α-ketoglutarate/isocitrate; and (iii) the percentage of viable sperm with an intact acrosome and α-hydroxyglutarate/α-ketoglutarate. Moreover, strong negative relationships were also detected between (i) intracellular calcium levels and citrate, citrate/malate and citrate/lactate (similarity score < −0.80); (ii) the percentage of sperm with high mitochondrial membrane potential and isocitrate/citrate, fumarate/succinate and α-ketoglutarate (similarity score < −0.70); and (iii) the percentage of viable sperm with an intact acrosome and α-ketoglutarate, isocitrate/citrate and isocitrate (similarity score < −0.70).

Finally, several strong relationships between different metabolites and IVF outcomes were found. Fertilisation rate at day 2 was the variable with the fewest associations with the other parameters, showing a negative relationship with isocitrate/citrate and fumarate/succinate (similarity score < −0.75), and a positive relationship with α-hydroxyglutarate, succinate/α-ketoglutarate and α-hydroxyglutarate/α-ketoglutarate (similarity score >0.50). Regarding embryo development parameters evaluated at day 6, several relevant relationships were also identified. Specifically, positive correlations were observed between (i) the percentage of total embryos and citrate, citrate/lactate and citrate/malate (similarity score >0.70); (ii) the percentage of morulae plus blastocysts and citrate/lactate, citrate and citrate/malate (similarity score >0.75); (iii) the percentage of morulae and citrate and isocitrate (similarity score >0.70); (iv) the percentage of hatching and hatched blastocysts and citrate, citrate/malate and citrate/lactate (similarity score >0.65); and (v) the developmental competency of fertilised oocytes and citrate and isocitrate (similarity score >0.70). On the other hand, negative correlations between (i) the percentage of morulae and α-hydroxyglutarate/isocitrate (similarity score < −0.75); (ii) the percentage of early blastocysts plus blastocysts and isocitrate/citrate and fumarate/succinate (similarity score < −0.75); (iii) the percentage of morulae plus blastocysts and α-ketoglutarate/isocitrate (similarity score < −0.75); and (iv) the developmental competency of fertilised oocytes and α-hydroxyglutarate/isocitrate (similarity score < −0.75) were also noticed.

Discussion

The precise catabolic pathway preferred by the sperm of each species is, in some cases, controversial. There are discrepancies between studies as, among other factors, the composition of semen extenders/preservation media differs. In effect, the availability of substrates directly influences the energetic pathway preferentially used by sperm cells. Particularly in pigs, while a recent study reported that non-capacitated sperm heavily rely upon mitochondrial Oxphos for ATP production[17], their metabolism has traditionally been assumed to be preferentially glycolytic. This assumption resides on the fact that, in porcine, (i) sperm catabolism of glucose produces lactate[26]; (ii) sperm contain mitochondria with few and practically nonvisible inner membrane crests[27]; (iii) sperm mitochondria crests are less condensed compared to their horse counterparts (whose metabolism is mainly oxidative)[28]; and (iv) a specific lactate dehydrogenase isozyme is present in sperm[29]. The results presented here, specifically the identification of lactate in sperm lysates and the absence of strong positive associations between sperm physiology and Krebs cycle intermediate metabolites, corroborate that glycolysis can be regarded as the main catabolic pathway used by non-capacitated pig sperm in high-quality, fertile semen samples. Interestingly, the data collected in this work confirm that the strategy of pig sperm to produce energy resembles to that of their human counterparts[13,14]. These metabolism similarities, together with the already reported analogy between species in terms of sperm physiology[24], opens a new range of possibilities in the study of the influence of metabolism on sperm function, fertility potential and contribution to embryo development using the pig as an animal model. For instance, fertility potential of sperm has been widely reported to be affected by their molecular composition, which mainly includes the proteome[2], lipidome[3], transcriptome[4] and metabolome[5]. Although the sperm metabolic profile is known to affect in vivo fertility outcomes in mammals[5], the exact way through which this element affects fertility is yet to be uncovered. The next step in this study was, therefore, to address how the energetic metabolic signature in mammalian sperm shapes their function and affects IVF outcomes.

Not only do sperm quality and function involve the evaluation of conventional sperm quality parameters such as motility, viability and morphology, but also that of other physiological processes, such as the acrosome reaction, mitochondrial activity and calcium homeostasis, among others[30]. The aforementioned processes are related to the sperm ability to fertilise an oocyte, therefore they have been traditionally used to estimate the reproductive performance of semen samples[30]. Characterising the factors that might be influencing sperm quality and function is thus crucial to understand the molecular mechanisms underlying the fertility of the male gamete. For this reason, the present work sought to address whether the energetic metabolic signature of sperm is related to their quality, function and fertilising ability. The positive relationship observed in the PLS model between lactate and citrate—but not other Krebs cycle metabolites—and sperm quality indicates that the main catabolic pathway in non-capacitated sperm samples of good quality (high motility and viability, and low incidence of morphological abnormalities) is glycolysis. This was further confirmed by multi-block data analysis, which revealed: (i) a positive relationship between glycolysis intermediates and the percentage of sperm with progressive motility, the percentage of viable sperm and the percentage of sperm with normal morphology; and (ii) a negative relationship between Krebs cycle intermediates and the percentage of sperm with progressive motility and the percentage of sperm with normal morphology. Although mitochondrial respiration is the most efficient source of ATP, glycolysis has also been associated with sperm of good quality in other mammalian species. In effect, glycolysis has been reported to be strongly related to sperm viability and motility in cattle[31], mice[13,32,33] and humans[34,35]. Yet, while glycolysis seems to be crucial for sustained sperm quality, additional studies should be carried out to evaluate if the mitochondrial activity also contributes to maintain sperm quality and even plays an essential role in the regulation of the events occurring in the female tract.

The PLS model showed that sperm function was negatively related to citrate, citrate/lactate, citrate/malate and isocitrate

(glycolysis metabolic markers), and positively related to α-hydroxyglutarate/isocitrate and α-ketoglutarate/isocitrate (Oxphos metabolic markers). From the multi-block analysis, the most interesting relationship identified was between Oxphos intermediates and intracellular calcium levels in sperm. The elevation of intracellular calcium levels is one of the first events of capacitation, a process physiologically induced in the female reproductive tract by the means of which sperm become fertilising competent[36]. In this sense, considering that capacitation should not occur in non-capacitating media, high levels of intracellular calcium in sperm could be understood as an indicator of poorer quality samples and, probably, reproductive outcomes as by the time of fertilisation, the status of the cell would not be appropriate. Thus, the positive association of α-ketoglutarate/citrate and α-hydroxyglutarate/isocitrate with intracellular calcium levels would line up with the previously set hypothesis: glycolysis rather than Oxphos is related with the best sperm quality traits. In addition, the association between intracellular calcium levels and Oxphos could also be considered to open a new research question: does porcine sperm metabolism switch during capacitation as already observed in rodents[37]? Ramió-Lluch et al.[38] already suggested that capacitation and the acrosome reaction are accompanied by a progressive increase of mitochondrial activity. In spite of this, as intracellular calcium levels are only the first step of capacitation, further research should determine the changes in the energetic metabolic signature occurring during sperm capacitation and how female fluids can affect sperm metabolism during these events. On the other hand, interestingly, the present work also found that the percentage of sperm with an intact acrosome was negatively related to glycolysis. Not much research about the role of sperm metabolism on physiological/spontaneous acrosome reaction has been conducted. A recent publication, however, reported that spontaneous acrosome reaction is independent from metabolic pathways in bovine sperm[39]. Whether spontaneous acrosome reaction in porcine sperm is also independent from metabolism is unknown. Since the present study was a first attempt to determine how metabolism affects sperm physiology, further studies are needed before a firm conclusion can be drawn.

The utility of metabolomic technologies to predict in vivo fertility from SP[40–47] or sperm[5,48] has widely been proved in several mammalian species. To the best of the authors' knowledge, nevertheless, no study in any mammalian species has looked into the relationship between the sperm metabolome and IVF outcomes. In general, the positive relationship observed in the PLS model between the first Krebs cycle metabolites (citrate, isocitrate and α-ketoglutarate) and IVF outcomes indicates that samples with the best IVF outcomes are highly associated to sperm whose main catabolic pathway is glycolysis. This would be in agreement with previous findings in mice, where the knockout of genes encoding for glycolytic-related proteins, such as Glyceraldehyde 3-phosphate dehydrogenase-S (*Gapdh*)[13], Phosphoglycerate kinase 2 (*Pgk2*)[33], Lactate dehydrogenase C (*Ldhc*)[32] or Cytochrome C (*CytCt*)[49], revealed that glycolysis rather than Oxphos is essential to preserve male fertility. Considering these results, whether sperm metabolism modulates the sperm ability to fertilise the oocyte and/or contributes to the subsequent embryo development was interrogated. The multi-block data integration showed a moderate, positive relationship between fertilisation rate and Oxphos, and a strong positive association between the total number of embryos at day 6 and glycolysis. This, together with the fact that the current study found a very strong association between fertilisation rate and the percentage of sperm with high mitochondrial membrane potential, would indicate that, at first glance, sperm using Oxphos as the principal catabolic pathway would have greater oocyte fertilising ability. Yet, oocytes fertilised by sperm preferentially using glycolysis appeared to produce more embryos at day 6. In a similar fashion, the developmental competency of fertilised oocytes was found to be positively associated to glycolysis-related metabolites. These results thus suggest that pre-implantation embryo development, rather than oocyte fertilisation, is closely influenced by sperm glycolysis. This idea would be supported by an additional finding of this study: sperm with the lowest levels of Oxphos metabolites are those that led to the highest percentages of the most developed embryos (i.e., percentages of morulae and blastocysts). How sperm metabolism can condition embryo development is unknown, but reactive oxygen species (ROS) might hold the key. Excessive ROS are known to affect sperm physiology through lipid peroxidation, motility reduction, apoptosis-like changes and even DNA damage[50]. Focusing on the latter, it has recently been reported that sperm DNA damage negatively affects embryo development in pigs[51]. Considering that ROS are mainly produced as a result of cellular respiration, one explanation for the data collected in the present study would be that sperm with excessive Oxphos activity could also bear greater DNA damage, which would compromise their IVF outcomes. Thus, sperm using glycolysis as their main energy source would probably contain less DNA damage, which would allow embryos to reach further pre-implantation stages. As this is only a hypothesis, the relationship between Oxphos/glycolysis and sperm DNA damage should be studied in the future.

The targeted metabolomics approach taken in this work allowed the characterisation of the main catabolic pathway in non-capacitated pig sperm, and addressed the relationship between their energetic metabolic status and fertility outcomes. This study also supported that glycolysis rather than Oxphos is used by sperm samples with good quality to produce energy. In addition, embryo development seemed to be tightly associated to glycolysis-related metabolites. These findings are a first steppingstone to explain how the sperm metabolome may influence fertility, as it shows that sperm metabolism has an impact on IVF outcomes. In addition, taking into consideration the similarities between pigs and humans in terms of the catabolic pathway preferred by their sperm, the results shown herein could be useful for the estimation of the success of IVF cycles using a non-invasive approach. Forthcoming studies should thus be focused on setting up specific metabolic biomarkers that could predict reproductive success.

Methods

Reagents. All reagents used in the present study were purchased from Sigma (Merck, Darmstadt, Germany) unless stated otherwise.

Animals. Semen samples were provided by an artificial insemination (AI) centre (Gepork S.L.; Masies de Roda, Spain), which follows the ISO certification (ISO-9001:2008), the EU Directive 2010/63/EU for animal experiments, the Catalan Animal Welfare Law, and the current regulation on Health and Biosafety issued by the Department of Agriculture, Livestock, Food and Fisheries, Regional Government of Catalonia, Spain. As ejaculates were commercially acquired from an AI centre and animals were not manipulated for the sole purpose of the present experiment, permission from an Ethics Committee was not required.

Ejaculates from healthy and sexually mature Pietrain boars (1–3 years old) were collected between June and July 2021 using the gloved-hand method. Immediately after collection, semen samples were diluted to a final concentration of 33×10^6 sperm/mL using a commercial extender (Vitasem LD, Magapor S.L., Zaragoza, Spain), and stored at 17 °C for 24 h. Upon arrival at the laboratory, the semen quality of doses was assessed to check if they met the conventional minimum requirements (sperm viability greater than 80% and motility greater than 70%).

On the other hand, ovaries were recovered from pre-pubertal gilts sacrificed for food purposes at a local abattoir (Frigorifics Costa Brava; Riudellots de la Selva, Girona, Spain).

Experimental design. Sixteen ejaculates meeting the quality standards and coming from the AI centre (each came from a separate boar; i.e., 16 boars) were included in the present study, and split into three aliquots. The first was used to assess sperm quality (which included sperm motility, viability and morphology) and function (which included acrosome integrity, intracellular calcium levels and mitochondrial membrane potential), and the second was used for IVF experiments. In brief, a

total of 650 oocytes were matured, fertilised, and both the fertilisation rate (day 2) and rates of embryo development at different pre-implantation stages (day 6) were recorded. Finally, the third aliquot was stored at –80 °C and later served to investigate sperm metabolomics through LC-MS/MS.

Sperm quality evaluation

Sperm motility. Semen samples were pre-warmed at 38 °C for 15 min, and 3 µL was placed into a Leja20 counting chamber (Leja Products BV; Nieuw-Vennep, The Netherlands). Following this, samples were evaluated under an Olympus BX41 microscope (Olympus; Tokyo, Japan) with a negative phase-contrast objective (Olympus 10× 0.30 PLAN objective, Olympus), through a computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System, ISAS V1.0; Proiser S.L.; Valencia, Spain). Two technical replicates were evaluated per sample, and at least 1000 sperm were examined in each replicate.

Two different parameters were recorded: the percentage of motile sperm, which considered those motile sperm whose average path velocity (VAP) was ≥10 µm/s; and the percentage of sperm with progressive motility, which included those motile sperm that exhibited a percentage of straightness (STR) ≥ 45%.

Sperm morphology. Sperm morphology was evaluated after dilution in 0.12% formaldehyde saline solution (PanReac AppliChem; Darmstadt, Germany; 1:1, v:v). Samples were observed under a phase-contrast microscope at ×1000 magnification (Nikon Labophot; Nikon; Tokyo, Japan), and 200 sperm cells were examined. Sperm cells were graded as morphologically normal, or with primary or secondary alterations[30]. The percentage of normal sperm was calculated from those without morphological alterations.

Sperm viability assessment. Sperm viability was assessed following the protocol of ref. [52], which uses SYBR-14 that stains sperm nuclei, and propidium iodide (PI) that only labels sperm having a compromised plasma membrane integrity. Briefly, semen samples were adjusted to a final concentration of 4×10^6 sperm/mL in 1× phosphate-buffered saline (PBS). Next, samples were incubated for 15 min at 38 °C with SYBR-14 (final concentration: 32 nM) and PI (final concentration: 7.5 µM). Stained cells were analysed using a CytoFLEX cytometer (Beckman Coulter; Brea, CA, USA), where SYBR-14 fluorescence was detected by the fluorescein isothiocyanate (FITC) channel (525/40), and PI using the PC5.5 channel (690/50). Both fluorochromes were excited with a 488-nm laser and no spill compensation was applied. Two technical replicates of at least 10,000 sperm were analysed at a constant flow rate, laser voltage and sperm concentration. The percentage of viable sperm corresponded to the SYBR-14$^+$/PI$^-$ population, after subtracting the percentage of debris particles in the analysis (Supplementary Fig 1).

Sperm function assessment. Sperm function was determined through the evaluation of intracellular calcium levels, acrosome membrane integrity and mitochondrial membrane potential using a CytoFLEX cytometer. Forward (FS) and side scatter (SS) were measured and linearly recorded for all particles. Subcellular debris and cell aggregates were excluded, and sperm events were positively gated through the adjustment of the analyser threshold on the FS channel. Finally, sperm-specific events were validated on the basis of FS/SS distributions (Supplementary Fig. 1).

Sperm intracellular calcium levels were assessed following ref. [53]; sperm were stained with Fluo3-AM (final concentration: 1.2 µM) and PI (final concentration: 5.6 µM) for 10 min at 38 °C in the dark. Fluo3 was detected through the FITC channel (525/40). The mean of Fluo3 fluorescence intensity per sperm (Fluo3$^+$/PI$^-$) was recorded and used for subsequent statistical analyses.

Acrosome membrane integrity was evaluated following the protocol of ref. [54], in which sperm were stained with PNA-FITC (final concentration: 1.2 µM) for 5 min at 38 °C in the dark, and then with PI (final concentration: 5.6 µM) for 5 min at 38 °C in the dark. PNA-FITC was detected by the FITC channel (525/40). The percentage of viable sperm with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$) was recorded and used for subsequent statistical analyses.

Mitochondrial membrane potential was evaluated following the protocol set by Ortega-Ferrusola et al.[55]. Sperm were incubated with JC-1 (final concentration: 750 nmol/L) for 30 min at 38 °C in the dark. In cells with high mitochondrial membrane potential, JC-1 aggregates and emits orange fluorescence, which is collected through the PE channel. On the contrary, in cells with low mitochondrial membrane potential, JC-1 is found in its monomeric form and generates green fluorescence, which is collected through the FITC channel. The percentage of sperm with high mitochondrial membrane potential was recorded and used for subsequent statistical analyses.

Oocyte maturation, in vitro fertilisation and embryo culture. Ovaries were transported to the laboratory in 0.9% NaCl supplemented with 70 µg/mL kanamycin at 38 °C. Cumulus oocyte complexes (COC) were retrieved from follicles and selected in Dulbecco's PBS (Gibco, ThermoFisher) supplemented with 4 mg/mL BSA. Only COCs exhibiting a complete and compact cumulus mass were included in the study.

For in vitro maturation of oocytes (IVM), TCM-199 (Gibco) supplemented with 0.57 mM cysteine, 0.1% (w:v) polyvinyl alcohol, 10 ng/mL human epidermal growth factor, 75 µg/mL penicillin-G potassium, and 50 µg/mL streptomycin sulphate was used. COCs were matured in groups of 40–50 in four-well multi-dishes (Nunc, ThermoFisher; Waltham, MS, USA) containing 500 µL of pre-equilibrated maturation medium supplemented with 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International B.V.; Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotropin (hCG; Veterin Corion; Divasa Farmavic S.A.; Gurb, Barcelona, Spain). After 20–22 h, oocytes were transferred into 500 µL fresh, pre-equilibrated IVM medium without hormones.

Next, mature oocytes were placed in 50-µL drops of pre-equilibrated IVF medium (Tris-buffered medium[56]) containing 1 mM caffeine. Semen samples were adjusted to 1000 sperm per oocyte in IVF medium and, thereafter, oocytes and sperm were co-incubated for 5 h in the incubator; a total of 40 oocytes per ejaculate were inseminated. Potentially fertilised oocytes were subsequently washed and transferred into 500 µL NCSU23 medium[57] supplemented with 0.4% BSA, 0.3 mM pyruvate and 4.5 mM lactate for embryo in vitro culture (IVC). After 2 days, cleaved embryos were counted to calculate fertilisation rates, and then transferred into NCSU23 medium supplemented with 0.4% BSA and 5.5 mM glucose. At day 6 post-fertilisation, the resulting embryos were classified following ref. [58] criteria. Specifically, the percentages of morulae, early blastocysts/blastocysts, hatching/hatched blastocysts and total embryos (sum of morulae, early blastocysts/blastocysts and hatching/hatched blastocysts) were evaluated. In addition, the sum of morulae, early blastocysts/blastocysts and hatching/hatched blastocysts was also determined to calculate the percentage of embryos in advanced stages. Finally, two different ratios were calculated: (i) the developmental potential at day 6, which corresponded to the percentage of morulae, early blastocysts/blastocysts plus hatched/hatching blastocysts divided by the percentage of 2–8 cell embryos; and (ii) the developmental competency of fertilised embryos, calculated as the ratio between the number of embryos at day 2 and those at day 6.

All procedures (oocyte IVM, IVF, and IVC) were carried out at 38.5 °C under a humidified atmosphere of 5% CO_2 in air.

Metabolomics

Sperm lysis. A total of 100 million sperm were lysed in 500 µL of lysis buffer (0.1% SDS 0.1% Triton in PBS). After samples were vortexed for 45 min at 4 °C, lysates were centrifuged at 18,000×*g* and 4 °C for 20 min. Supernatants were recovered and stored at −80 °C until LC-MS/MS analysis was carried out. Two technical replicates per semen sample were processed. In addition, and in order to prepare the blank, all the protocols were applied in parallel to four replicates that did not contain sperm samples.

LC-MS/MS analysis. Cell lysates were analysed by adapting a previously reported method for the quantification of polycarboxylic acids[59]. The method involved a derivatisation with o-benzylhydroxylamine, a liquid–liquid extraction with ethyl acetate and LC-MS/MS detection using a selected reaction monitoring mode. A LC-MS/MS system consisting of an Acquity UPLC instrument (Waters Associates, Milford, MA, USA) coupled to a triple quadrupole (TQS Micro, Waters) mass spectrometer was used for the analysis. Lactic acid, citric acid, isocitric acid, α-ketoglutarate, succinic acid, fumaric acid, malic acid, acetoacetate and α-hydroxyglutarate were determined. In addition to the concentration of each metabolite, nine ratios between metabolites with potential information about enzyme activity were calculated. MassLynx software V4.1 (Waters Associates) was used for peak integration and data management.

Statistics and reproducibility

Data analysis. Data preprocessing and statistical analyses were conducted using the R software (version 4.2.0). The sample size for linear regression was calculated using the "pwr.f2.test" function from the "pwr" R package[60]. Missing values were replaced by half of the minimum value within the dataset. The Shapiro-Wilk test was used to assess normality. The metabolomics dataset was log-transformed before modelling.

Sperm physiology and in vitro fertility parameters were classified into three main blocks: sperm quality, sperm function and IVF outcomes, and were analysed separately. Each group was log-transformed and scaled prior to running PCA for dimensionality reduction purposes. PCA disposes an orthogonal projection onto a lower dimensional subspace, which captures the majority of the variance of the dataset[61]. Then, variables of each block were projected onto a few principal loading vectors independently, condensing most of the variability of the original data[62]. Score values from the first PC of each block were utilised as a reduced-dimension feature vector in the response block (Y-block), predicted in function of the metabolomics set (X-block) using a multivariate PLS regression. The generation of the PLS model was carried out through the root mean square error of prediction as metric in a repeated double-cross-validation framework[63], including a recursive ranking based on variable importance in projection and sequential backward feature removal[64]. The whole operation was repeated 20 times for improved coverage of inner and outer segments and modelling performance. The model performance was assessed by means of a permutation test of 500 iterations between permuted models, with a random assignation of the observations, and the actual model obtained. Furthermore, linear models were run on metabolic data using the reduced-dimension feature vectors as response. The Benjamini–Hochberg procedure was carried out on all analyses to control the false discovery rate

(FDR)[65]. Only FDR-corrected *P* values lower than 0.05 were considered as statistically significant.

Multi-block data integration. Integration of multiple datasets measured on the same observations was conducted utilising the N-integration with Projection to Latent Structures model[66]. This model was built to assess multi-block correlations between sperm quality, sperm function, IVF outcomes, and metabolomic blocks from the same observational units, using the mixOmics R package v 6.18.1[67]. A pair-wise similarity matrix was constructed from the two correlated latent components obtained through the projection to latent structures method. A relevance network graph was created to describe connections between the four datasets, based on the rule of similarity score ≥0.3[68].

Reporting summary. Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The datasets used and/or analysed during this study are available as Supplementary Data.

Code availability

R studio V4.2.0 was used for all analyses. The sample size for linear regression was calculated using the "pwr.f2.test" function from the "pwr" R package. Repeated double-cross-validation PLS regressions were run using the "MUVR" R package (available at https://gitlab.com/CarlBrunius/MUVR). The "mixOmics" R package was used for PCA and the integration of multi-block data was conducted through "block.pls" function. Codes for sample size calculation, PLS regressions, linear models, and multi-block data integration are available on GitHub (https://github.com/Francisco-madrid-gambin/CodeSharingTechnoSperm).

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Author contributions

Conceptualisation: Y.M.-O., F.M.-G., M.L., O.P. and M.Y.; methodology: Y.M.-O., F.M.-G., M.L., A.G.-G. and N.H.; formal analysis and investigation: Y.M.-O., F.M.-G. and M.L.; writing—original draft preparation: Y.M.-O. and F.M.-G.; writing— review and editing: M.L., A.G.-G., O.P. and M.Y.; funding acquisition: O.P. and M.Y.; supervision: O.P. and M.Y. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Paper VI

Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs.

Yentel Mateo-Otero, Marc Llavanera, Sandra Recuero, Ariadna Delgado-Bermúdez, Isabel Barranco, Jordi Ribas-Maynou, Marc Yeste.

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Biological Research

RESEARCH ARTICLE Open Access

Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs

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Abstract

Background: The assessment of sperm DNA integrity has been proposed as a complementary test to conventional mammalian semen analysis. In this sense, single-strand (SSB) and double-strand (DSB) DNA breaks, the two types of sperm DNA fragmentation (SDF), have been reported to have different aetiologies and to be associated to different fertility outcomes in bovine and humans. Considering that no studies in porcine have addressed how SDF may affect sperm quality and fertility outcomes, the present work aimed to determine the impact of global DNA damage, SSB and DSB on sperm quality and in vitro fertilising ability. To this end, 24 ejaculates (one per boar) were split into three aliquots: the first was used to assess sperm quality parameters through a computer-assisted sperm analysis (CASA) system and flow cytometry; the second was used to perform in vitro fertilisation, and the third, to evaluate sperm DNA integrity using alkaline and neutral Comet assays.

Results: The results showed that global DNA damage negatively correlates ($P < 0.05$) with normal sperm morphology ($R = -0.460$) and progressive motility ($R = -0.419$), and positively with the percentage of non-viable sperm ($R = 0.507$). Multiple regression analyses showed that non-viable sperm were related to SSB ($\beta = -0.754$). In addition, while fertilisation did not seem to be affected by sperm DNA integrity, global DNA damage, DSB and SSB were found to be correlated to embryo development outcomes. Specifically, whereas global DNA damage and DSB negatively affected ($P < 0.05$) the later preimplantation embryo stages (percentage of early blastocyst/blastocyst D6: for global DNA damage, $R = -0.458$, and for DSB, $R = -0.551$; and percentage of hatching/hatched blastocyst D6: for global DNA damage, $R = -0.505$, and for DSB, $R = -0.447$), global DNA damage and SSB had a negative impact ($P < 0.05$) on the developmental competency of fertilised embryos ($R = -0.532$ and $R = -0.515$, respectively). Remarkably, multiple regression analyses supported the associations found in correlation analyses. Finally, the present work also found that the inclusion of Comet assays to the conventional sperm quality tests improves the prediction of blastocyst formation ($AUC = 0.9021$, $P < 0.05$), but not fertilisation rates ($P > 0.05$).

Conclusion: Considering all these findings, this work sets a useful model to study how SDF negatively influences fertility.

Keywords: Sperm DNA damage, Embryo development, Oocyte fertilisation, Porcine

Background

Over the last decades, research on the improvement of assisted reproductive techniques (ART) has gained much relevance due to the decreased human fertility rates and the improvement of profitability in livestock reproduction [1–3]. In this realm, infertility has been typically considered as a multifactorial pathological condition

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involving the combined effect of male and female factors in equal parts [4]. Focusing on the male factor, mounting evidence indicates that the assessment of conventional sperm quality parameters does not efficiently predict the efficiency of ART [5, 6]. For this reason, more complex tests, including the evaluation of sperm functionality by flow cytometry [7] or the assessment of sperm DNA integrity [8], have been developed. Despite that, controversial results reported by some clinical studies have led scientific societies to pronounce different opinions about the suitability of including these advanced tests into the human semen routine analysis [9–13].

Sperm DNA fragmentation (SDF) is a genotoxic insult occurring in response to intrinsic or extrinsic oxidative stress, as a result of chromatin remodelling during spermiogenesis or due to enzymatic activity and apoptotic-like processes [14, 15]. Recently, the use of advanced methods that allow discriminating different types of sperm DNA damage has shown that single-strand (SSB) and double-strand (DSB) DNA breaks may have different aetiologies and may lead to reproductive consequences [16]. On the one hand, SSB are an oxidative-related DNA damage mainly caused by oxidative stress, which is produced by the imbalance between reactive oxygen species (ROS) and antioxidants. The ROS are highly-reactive small radicals that interact with nitrogenized bases of the DNA, forming DNA adducts such as 8-hydroxy-2′deoxyguanosine (8OHdG), which are excised and generate a SSB [17]. This effector mechanism usually leads to an extensive DNA damage distributed alongside the sperm genome, both in toroidal and toroid linker regions, resulting in lack of pregnancy or an increase of conception time [16, 18]. On the other hand, DSB have been shown to be highly localised at the toroid linker regions and is probably triggered by the enzymatic activity occurring at meiotic or post-meiotic stages. Remarkably, DSB has been reported to increase the risk of implantation failure and miscarriage, and is associated to low embryo quality [18–22].

To date, many methods with different molecular basis have been developed to evaluate sperm DNA fragmentation, the most used ones being (1) the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), (2) the sperm chromatin structure assay (SCSA), (3) the sperm chromatin dispersion (SCD) and (4) the Comet assay. Despite the high standardization of TUNEL, SCSA and SCD tests, their major drawback is their inability to separately evaluate SSB/DSB [16, 21]. Contrarily, while the Comet assay is a less standardized method with wide variations between laboratories, it can be performed under alkaline or neutral pH to specifically discriminate between SSB and DSB [16, 21]. Despite the high amount of studies conducted in mammalian species using different SDF methods, their different molecular basis and the lack of consensus regarding the cut-off values have led to controversial conclusions about their usefulness in ART. In effect, while some authors find a negative relationship between DNA fragmentation and fertility [23, 24], others do not observe such an association [25, 26]. Yet, a recent meta-analysis conducted in a substantially high number of human patients showed that these discrepancies may not only reside in the method of analysis of DNA fragmentation, but could also be explained by the different association between SDF and in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) [27]. Thus, while there is a consensus on the detrimental impact of SDF on natural pregnancies and IVF outcomes, this is not the case of ICSI [27]. This difference is likely to be explained by the technical differences between ICSI and IVF, as the former involves the selection of a single spermatozoon based on its motility and morphology, traits that have been shown to be negatively correlated to DNA damage [28, 29]. To bring light into the topic, a recent systematic review pointed out that oxidative DNA damage induced in sperm from different mammalian species has an adverse effect on IVF and ICSI embryos [30]. Similarly, inconsistent data about the impact of SDF on sperm quality have been reported. Indeed, whereas some observed close associations between SDF and seminogram parameters [19, 28, 31–37], others did not [38, 39].

Besides studies conducted in humans, the impact of SSB and DSB on fertility outcomes has been scarcely evaluated in other mammalian species. Establishing the effects and the potential relationship between the different types of DNA damage and sperm quality parameters, fertilisation and even embryo development could, however, open the possibility of using animal models to evaluate the precise genotoxic DNA damage induced by extrinsic factors, their effector mechanism and their impact on fertility rates [40]. In this sense, porcine species has been previously proposed as a suitable animal model for the study of sperm capacitation, fertilisation and male infertility [41]. While a recent work carried out by our research group characterised the two types of DNA breaks in pig sperm [42], no study has explored their potential relationship to IVF outcomes. The aim of the present study, therefore, was to determine the effects of SSB and DSB on: (i) sperm quality parameters; (ii) oocyte fertilisation; and (iii) embryo development.

Results

Relationship of global DNA damage, SSB and DSB with sperm quality parameters

The first aim of the present study was to evaluate the potential relationship between SDF and sperm quality

parameters, in terms of sperm morphology, motility and viability. To this end, the global DNA damage was calculated as Olive Tail moment (OTM) from the alkaline Comet, DSB were evaluated using the OTM from neutral Comet and, finally, SSB were calculated by subtracting the neutral Comet OTM from the alkaline Comet OTM. Next, Spearman correlations were calculated with each of these parameters (Fig. 1A). Moreover, because a strong correlation between global DNA damage and SSB was observed ($R = 0.925$; $P = 0.925$), multiple regression analyses including SSB, DSB and morphology, motility or viability variables were conducted.

Regarding sperm morphology, positive correlations ($P < 0.05$) between the percentage of sperm with abnormal morphology and global DNA damage and SSB were observed ($R = 0.460$ and $R = 0.403$, respectively). Regarding sperm motility, only global DNA damage was found to exhibit a negative correlation ($P < 0.05$) with the percentage of sperm with progressive motility ($R = -0.419$) and specific sperm motility kinematic parameters, including straight-line velocity (VSL), average path velocity (VAP), percentage of linearity (LIN) and motility parameter wobble (WOB) ($R = -0.424$, $R = -0.421$, $R = -0.397$, $R = -0.430$, respectively). Finally, the percentage of non-viable sperm was positively correlated ($P < 0.05$) with global DNA damage, DSB and SSB ($R = 0.507$, $R = 0.439$ and $R = 0.394$, respectively). Multiple regression analyses showed no association between SSB or DSB and morphology or motility ($P > 0.05$), but did find an association between SSB and sperm viability ($\beta = -0.754$; $P = 0.019$).

Relationship of global DNA damage, SSB and DSB with IVF outcomes

This study also explored the effect of SDF on oocyte fertilisation and embryo development. To this end, Spearman correlations of global DNA damage, SSB and DSB with IVF outcomes were calculated (Fig. 1B).

First, no correlation ($P > 0.05$) between fertilisation rate on day 2 and any of the SDF indices evaluated was found. On the contrary, several correlations between embryo development and the different SDF types were observed. Specifically, the total number of embryos on day 6 was negatively correlated ($P < 0.05$) to both global DNA damage and SSB ($R = -0.535$ and $R = -0.526$, respectively). In addition, the different SDF types were also observed to have an influence on specific embryo stages. In effect, both global DNA damage and DSB exhibited the same pattern, showing a negative correlation ($P < 0.05$) with the percentages of early blastocysts/blastocysts and hatching/hatched blastocysts (for global DNA damage: $R = -0.468$ and $R = -0.505$, respectively; for DSB: $R = -0.551$ and $R = -0.477$, respectively), but not with the percentages of morula ($P > 0.05$). On the other hand, SSB were not found to correlate ($P > 0.05$) with any of

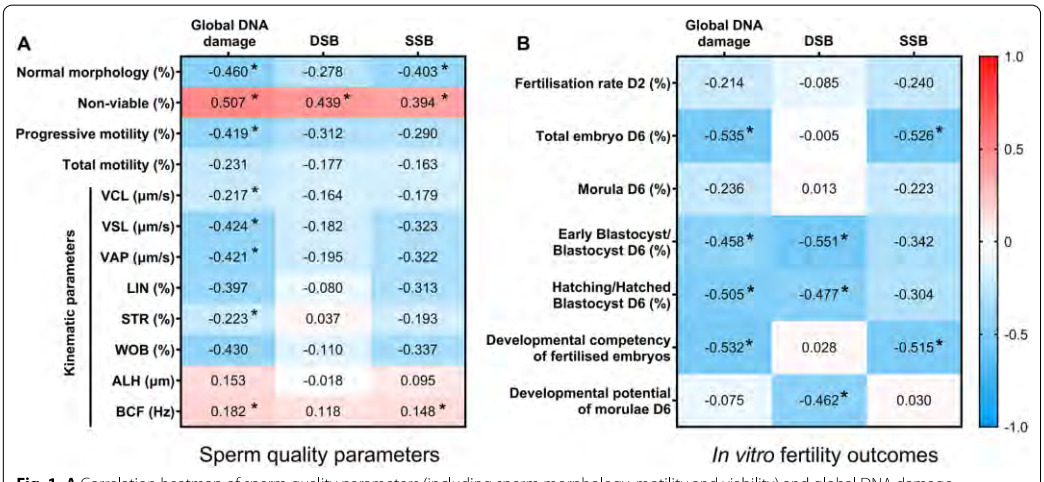

Fig. 1 **A** Correlation heatmap of sperm quality parameters (including sperm morphology, motility and viability) and global DNA damage, double- (DSB) and single-SDF (SSB). **B** Correlation heatmap of in vitro fertility outcomes evaluated on day 2 (D2) and Day 6 (D6) and global DNA damage, DSB and SSB. Semen samples of 24 AI-boars split in three aliquots: the first was used to assess sperm quality after semen samples arrived at the laboratory, the second was used to perform in vitro fertility procedures, and the third was used to evaluate alkaline and neutral Comet. The colour saturation of red to blue represents the Pearson correlation coefficients (R) from 1 to − 1, respectively. Significant correlations ($P < 0.05$) are marked with *

the embryo stages on day 6. Considering the correlation found between global and DNA damage and SSB (shown in "Relationship of global DNA damage, SSB and DSB with sperm quality parameters" section), multiple linear regression analysis were subsequently conducted including SSB, DSB, day 2 fertilization rate and day 6 embryo outcomes (total number of embryos, morulae, early blastocysts/blastocysts and hatching/hatched blastocysts). The analysis confirmed that no association between fertilization rate on day 2 and SSB or DSB existed ($P > 0.05$), and showed an association between SSB and total number of embryos on day 6 ($\beta = -0.141$; $P = 0.010$), between DSB and early blastocysts/blastocysts ($\beta = -0.042$; $P < 0.001$), and between DSB and hatching/hatched blastocysts ($\beta = -0.140$; $P = 0.018$).

To evaluate the developmental potential of morulae, the percentage of early blastocysts/blastocysts plus hatched/hatching blastocysts was divided by the percentage of morulae. DSB were negatively correlated with developmental competency ($R = -0.418$; $P = 0.023$), but neither global DNA damage nor SSB showed such a relationship ($P > 0.05$). The multiple regression analysis also showed the association of this parameter to DSB ($\beta = -0.890$; $P = 0.044$), but not to SSB ($P > 0.05$), with the developmental potential of morulae.

Finally, the developmental competency of fertilised embryos was calculated as the ratio between the total number of embryos on day 6 and the total number of embryos on day 2. Whereas global DNA damage and SSB were found to negatively correlate ($P < 0.05$) with the embryo developmental rate ($R = -0.532$ and $R = -0.515$, respectively), DSB did not ($P > 0.05$). The results obtained from the multiple regression analysis were similar ($P > 0.05$ for DSB; $\beta = -0.065$ and $P = 0.042$ for SSB).

Prediction of in vitro fertility outcomes through conventional sperm quality parameters and Comet

The last aim of this study was to evaluate whether the inclusion of alkaline and neutral Comet tests to the conventional semen analysis (which comprises the assessment of sperm morphology, motility, and viability) improved the prediction of IVF outcomes, specifically, fertilisation rate on day 2 and percentage of total blastocysts on day 6.

First, semen samples were divided by the median of fertilisation rate on day 2 in two groups: low (ranging 20.0–29.3%, n = 12) and high (ranging 32.5–63.4%, n = 12) fertilisation rate. Then, a Receiver Operating Characteristic (ROC) curve was elaborated for each sperm quality parameter (Table 1). The ROC curve analysis showed that only the percentage of total motility was able to predict the fertilisation rate on day 2 ($P < 0.05$), showing a good discriminant value with an Area Under the Curve (AUC) of 0.8750. In addition, none of the Comet assays exhibited a significant AUC ($P > 0.05$). Following this, principal components were extracted to elaborate a combination of all the parameters (sperm motility, morphology, viability and alkaline and neutral Comet), and ROC curve analysis was redone for the first component. The combination of all parameters, however, showed no significant AUC (Fig. 2A).

Next, semen samples were categorized in two groups considering the median of the percentage of total blastocysts on day 6 (calculated as the sum of the percentage of early blastocysts/blastocysts and that of hatched/hatching blastocysts): low (ranging 2.9–10.0%, n = 13) and high (ranging 11.4–29.0%, n = 11). Then, a ROC curve analysis for each sperm quality parameter was run (Table 2). In this case, the percentages of total motile and viable sperm exhibited a good discriminant predictive value with an AUC of 0.8392 and 0.8671, respectively ($P < 0.05$). Moreover, a tendency ($P = 0.0597$) for the AUC of both sperm morphology and progressive motility was found, with an associate AUC of 0.77273 in both cases. Additionally,

Table 1 Receiver operating characteristic (ROC) for each sperm quality parameter to predict fertilisation rate on day 2

	AUC (95% CI)	*P* value	Cut-off value (%)	Sensitivity (95% CI)	Specificity (95% CI)	ODDs ratio
Morphology	0.5556 (0.3174–0.7938)	0.6442	94.53	58.33% (31.95–80.67%)	66.67% (39.06–86.19%)	1.750
Total motility	0.8750 (0.7246–1.000)	0.0018	88.61	75.00% (46.77–91.11%)	91.67% (64.61–99.57%)	9.000
Progressive motility	0.5625 (0.3211–0.8039)	0.6033	78.14	41.67% (19.33–68.05%)	91.67% (64.61–99.57%)	5.000
Viability	0.6806 (0.4476–0.9135)	0.1333	90.03	66.67% (39.06–86.19%)	75.00% (46.77–91.11%)	2.667
OTM alkaline-neutral Comet	0.5833 (0.3458–0.8208)	0.4884	10.80	25.00% (8.894–53.23%)	91.67% (64.61–99.57%)	3.000
OTM neutral Comet	0.5486 (0.3081–0.7892)	0.6861	3.658	33.33% (13.81–60.94%)	91.67% (64.61–99.57%)	4.000
Combination (Component 1)	0.5903 (0.3543–0.8261)	0.5529	0.5590	25.00% (8.89–53.35%)	91.67% (64.61–99.57%)	3.000

AUC area under the curve; *CI* confidence interval; *OTM* olive tail moment

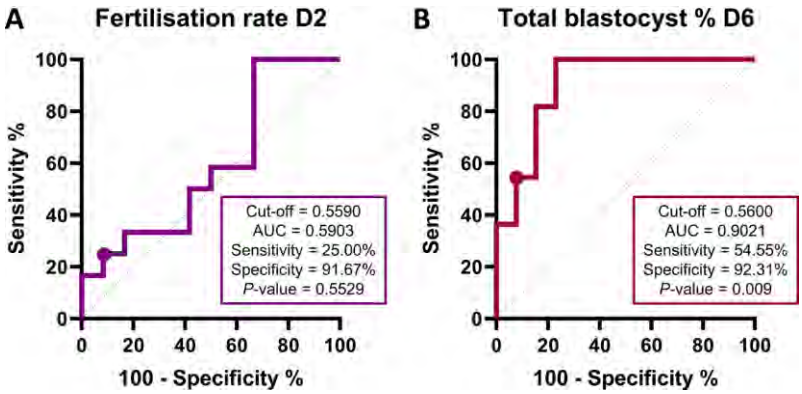

Fig. 2 Receiver operating characteristic (ROC) curve analysis for fertilisation rate on day 2 (**A**) and percentage of total blastocyst on day 6 (**B**). They show the ability of conventional sperm quality parameters combined with Alkaline and Neutral Comet assay to discriminate the fertilisation rate and the percentage of total blastocysts on day 6. *AUC* area under the curve

Table 2 Receiver operating characteristic (ROC) for each sperm quality parameter to predict the total blastocyst percentage on day 6

	AUC (95% CI)	P value	Cut-off value (%)	Sensitivity (95% CI)	Specificity (95% CI)	ODDs ratio
Morphology	0.7273 (0.5077–0.9468)	0.0597	94.18	81.82% (52.30–96.77%)	76.92% (49.74–91.82%)	3.545
Total motility	0.8392 (0.6734–1.000)	0.0050	91.39	45.45% (21.27–71.99%)	92.31% (66.69–99.61%)	5.909
Progressive motility	0.7273 (0.5242–0.9304)	0.0597	80.19	36.36% (15.17–64.62%)	92.31% (66.69–99.61%)	4.727
Viability	0.8671 (0.7251–1.000)	0.0024	91.37	63.64% (35.38–84.83%)	92.31% (66.69–99.61%)	8.273
OTM alkaline—neutral Comet	0.7273 (0.5175–0.9370)	0.0597	11.67	45.45% (21.27–71.99%)	92.31% (66.69–99.61%)	5.909
OTM neutral	0.8042 (0.6024–1.000)	0.0117	2.72	90.91 (62.26–99.53%)	84.62 (57.77–97.27%)	5.909
Combination (Component 1)	0.9021 (0.7773–1.000)	0.0009	0.56	54.55 (28.01–78.73%)	92.31 (66.69–99.61%)	7.091

AUC area under the curve; *CI* confidence interval; *OTM* olive tail moment

although only the neutral Comet assay showed a significant AUC ($P<0.05$), displaying a good discriminant value to predict the percentage of total blastocysts on day 6 with an AUC of 0.8042, a tendency ($P=0.0597$) for the AUC of the alkaline-neutral OTM was observed (AUC = 0.7273). When principal components were extracted to address whether a combined model of all the parameters predicted the percentage of total blastocysts on day 6, an excellent discriminant value for the first component, with an AUC of 0.9021, was found ($P<0.05$; Fig. 2B).

Discussion

Sperm DNA fragmentation has been shown to have a great impact on natural fertility outcomes [21, 27] and sperm quality parameters [28] in humans. In porcine, although it has been reported that global DNA damage affects litter size [43, 44], the effects of specific DNA breaks on sperm fertilising ability and embryo development have not been addressed. To this end, the present work aimed to explore the relationship between global DNA damage, SSB and DSB evaluated using the Comet assay and sperm quality parameters and IVF outcomes. The results of the present study indicate that: (i) the incidence of global DNA breaks correlates with sperm quality, assessed in terms of sperm morphology, motility and viability; (ii) SDF is not correlated to the sperm ability to fertilise oocytes; (iii) global DNA damage and DSB may disturb late pre-implantation embryo development, and global DNA damage and SSB have a negative impact on embryo developmental competency from day 2 to day 6; and (iv) the inclusion of Comet assays to the conventional spermiogram parameters improves the prediction of IVF success, specifically blastocyst formation.

There is conflicting evidence about the impact of sperm DNA breaks on sperm quality in humans [28, 31–36, 38, 39]. In porcine, only one study from our group addressed this, finding no correlation between sperm quality and neutral Comet OTM and only a weak correlation between sperm kinematic parameters and Alkaline Comet OTM

[45]. Moreover, no study has investigated the relationship of global DNA damage, SSB and DSB with sperm quality in livestock. The present report found a positive correlation between the incidence of SSB and the percentage of morphologically abnormal sperm, which were not confirmed by the multiple regression analysis. Previous studies performed in humans [28, 34–37] and cattle [46] showed increased levels of sperm DNA breaks in semen samples with a high percentage of sperm with morphological abnormalities. Yet, it is worth mentioning that none of the aforementioned studies evaluated the correlation between sperm morphology and SSB or DSB independently. For this reason, the present study is the first suggesting a possible positive relationship between SSB and sperm morphological abnormalities. A direct cause-effect, however, was not observed through the multiple regression analysis, thus suggesting that a third player influencing those alterations should not be discarded. In addition, the present work also assessed the relationship between sperm motility parameters and DNA integrity, finding a negative correlation between the incidence of global DNA breaks, the percentage of sperm with progressive motility and several motility kinematic parameters. These results are in agreement with a previous work in pig sperm, in which log-transformed DNA fragmentation index assessed through SCSA negatively correlated with sperm motility [43]. However, unlike other studies in which SSB, but not localised DSB, negatively influenced progressive motility in humans [19], the results of this study found no relationship between any of the specific DNA break types and motility parameters. The relationship found between global DNA damage and motility in our study is, nevertheless, in accordance with previous reports in humans [31–33]. Again, a lack of cause-effect association between SSB and DSB and sperm motility was observed, suggesting that both parameters could be altered upon exposure to a third causative mechanism, which may be, for instance, oxidative stress [47]. Finally, the current work also identified a negative correlation between the incidence of global DNA breaks, SSB and DSB and the percentage of non-viable sperm. Although, to the best of our knowledge, no previous study addressed whether the specific DNA breaks are related to sperm viability, earlier reports in humans found a strong negative correlation between DNA fragmentation and this sperm parameter [48]. In this case, SSB were found to be associated to the percentage of non-viable sperm in a multiple regression analysis, evidencing that cell death is closely related to DNA damage.

The impact of SDF on fertility has been extensively studied in humans [23, 24, 27] and DNA fragmentation evaluated with SCSA has been reported to be negatively related to farrowing rate and litter size in productive species [43, 49, 50]. Hence, after investigating the link between DNA breaks and conventional spermiogram parameters, we hypothesised that the different types of SDF could also lead to different outcomes after IVF. Our results showed that, while fertilisation rate on day 2 was not caused by or related to sperm DNA damage, global DNA breaks and SSB negatively influenced the number of embryos obtained on day 6. These findings indicate that, while DNA integrity does not affect the sperm ability to fertilise oocytes, it may compromise embryo development, as it has been already posited before in bovine [51] and human [34, 52]. Indeed, a negative relationship between global DNA breaks and SSB and developmental competency of fertilised embryos was found herein, suggesting that both global DNA damage and extensive SSB in sperm strongly compromise the embryo ability to develop after very early embryo stages. Importantly, not only were global DNA breaks and DSB found to negatively affect the percentages of early blastocysts/blastocysts and hatching/hatched blastocysts, but DSB was also seen to influence negatively the developmental potential of morulae. These results are in agreement with previous reports in humans, mice, cattle and goats, in which embryos produced with sperm containing DSB showed delays in their developmental kinetics and, ultimately, lower implantation rates and miscarriage within the first trimester [18–20, 53, 54]. Previous reports in mice proposed that extensive sperm DSB may probably exceed the oocyte repair capacity; consequently, paternal DNA replication may be delayed leading to embryonic developmental arrest [20]. Another hypothesis would be that sperm DSB could potentially lead to chromosome aberrations and mutations during early embryonic development, which could lead to cell death, thus inhibiting embryo development [53, 55, 56]. Indeed, the negative impact of DSB on morula developmental competency reported in the present work may be explained by the fact that it is not until the morula stage when chromosome aberrations trigger G1/S and G2/M checkpoints [57], which are likely to activate apoptotic mechanisms and avoid blastocyst formation [58]. Interestingly, as it has been already observed in human embryos [19], the present study also found that SSB do not seem to have an impact on embryo kinematics in porcine; however, further studies using time-lapse technologies are needed to confirm these observations. As previously hypothesised in humans, this could result from the capacity of zygotes to repair SSB since the complementary DNA strand is present [19]. Either way, the present study reinforces the idea that DSB have a dramatic, detrimental impact on mammalian embryo development and, for this reason, their assessment may contribute to increasing the efficiency of ART procedures.

The assessment of sperm DNA damage has been extensively proved to have a strong predicting ability for human fertility [27, 59]. Regarding the tests evaluating that damage, TUNEL, SCSA and Comet assays have been shown to be the most powerful [60, 61]. The use of more advanced methods, such as the Comet assay, however, is interesting due to: (i) its inherent ability to discriminate DSB and SSB [42], (ii) its high reproducibility and sensitivity [39, 62]; and (iii) its ability to equally detect breaks in protamine and histone-bound chromatin [39]. Considering this and the results presented herein, this work also evaluated whether including Comet assay to the conventional semen analysis could improve fertility prediction. Our data showed that, while the Comet assay was unable to predict fertilisation rate on day 2, including the evaluation of sperm DNA integrity through this technique to the traditional spermiogram had an additive effect, depicting an excellent discriminant value for predicting the percentage of blastocysts on day 6. This did not come as a surprise as we observed a relationship between the different types of SDF and embryo development parameters on day 6, but not between SDF and fertilisation rate on day 2. In addition, while this is the first report including the Comet assay to the routine semen analysis in livestock, previous studies in pigs [43, 50] and cattle [49, 63–66] already traced the clinical significance of other sperm DNA fragmentation assays. The present work, therefore, confirms using an animal model that routine testing of DNA integrity improves assisted reproduction outcomes, as previously advised for humans [27]. Also, the establishment of this relationship in porcine enables future studies assessing the effects of different putative treatments or genotoxic compounds on sperm DNA integrity, thus helping in the prevention and diagnosis of human reproductive disorders. In addition, future studies including the use of ICSI in animal models may help address whether sperm DNA fragmentation status differently affects IVF and ICSI outcomes.

Conclusions

Sperm DNA damage has been previously found to influence fertility in mammalian species. Yet, no report has exhaustively evaluated the relationship of sperm SSB and DSB with sperm quality parameters, oocyte fertilisation and embryo development in porcine. The results of the present work concluded that SSB and DSB have a different impact on pig sperm quality parameters. Moreover, although sperm DNA damage does not seem to be related to the sperm ability to fertilise the oocyte, the present report evidences that while SSB are correlated to the amount of embryos observed on day 6, DSB compromise the percentage of embryos reaching the blastocyst stage. Importantly, our data support that the combination of the two Comet variants with conventional sperm quality parameters achieves very high discriminant value for embryo development outcomes. For all these reasons, this work sets a useful model to study how genotoxic agents inducing sperm DNA fragmentation affect fertility.

Materials and methods

Reagents

Unless stated otherwise, all reagents used in the present study were of analytical grade and purchased from Sigma (Merck, Darmstadt, Germany). Fluorochromes were acquired from ThermoFisher Scientific (Waltham, MA, USA).

Animals and samples

All semen samples used in the present study were provided by a local farm (Gepork S.L.; Masies de Roda, Spain), which follows the ISO certification (ISO-9001:2008). All the procedures that involved animals were performed by the AI centre in accordance with the EU Directive 2010/63/EU for animal experiments, the Animal Welfare Law issued by the Regional Government of Catalonia, and the current regulation on Health and Biosafety issued by the Department of Agriculture, Livestock, Food and Fisheries, Generalitat de Catalunya, Spain. As no animal was manipulated to conduct the present experiment, since ejaculates were commercially acquired from a local farm (AI-centre), no permission from an Ethics Committee was required.

Ejaculates from healthy and sexually mature Pietrain boars (1–3 years old) were collected using the gloved-hand method. Immediately after collection, semen samples were diluted to a final concentration of 33×10^6 sperm/mL using a commercial extender (Vitasem LD, Magapor S.L., Zaragoza, Spain) and stored at 17 °C for 24 h.

Experimental design

Twenty-four ejaculates from 24 boars (one ejaculate per boar) were used to conduct the analyses described below. Each ejaculate, considered as a biological replicate, was split into three aliquots: the first was used to assess sperm quality, in terms of sperm motility, morphology and viability; the second was intended to IVF; and the third aliquot was stored at − 80 °C until alkaline and neutral Comet assays were carried out.

Evaluation of sperm quality

Sperm motility

Sperm motility was assessed through a computer-assisted sperm analysis (CASA) system (Integrates Sperm Analysis System, ISAS V1.0; Proiser S.L.; Valencia, Spain) and

Olympus BX41 microscope (Olympus; Tokyo, Japan) with a negative phase contrast field (Olympus 10 × 0.30 PLAN objective, Olympus). Semen samples were incubated for 15 min at 38 °C, and 5 µL of each sample were analysed in a pre-warmed Leja20 counting chamber (Leja Products BV; Nieuw-Vennep, The Netherlands). Two technical replicates were examined, evaluating 1000 sperm per replicate.

Several sperm velocity parameters were recorded: VSL, VAP, curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), LIN, percentage of straightness (STR) and WOB. Total motility and progressive motility were also recorded, and sperm were considered motile when VAP was ≥ 10 µm/s, and progressively motile when STR was over 45%.

Sperm morphology

After diluting semen samples with 0.12% formaldehyde in saline solution (PanReac AppliChem; Darmstadt, Germany; 1:1, v:v), sperm morphology was analysed under a phase-contrast microscope at 1000 × magnification (Nikon Labophot; Nikon; Tokio, Japan). Two hundred sperm cells were counted and those without morphology alterations were considered as normal. Moreover, primary and secondary alterations were recorded [67].

Sperm viability assessment

The LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) following the protocol of Garner and Johnson [68] was used to evaluate plasma membrane integrity. This kit includes SYBR-14, which stains the nuclei of all sperm, and propidium iodide (PI), which only stains those of sperm that have lost their plasma membrane integrity. In brief, semen samples were diluted to a final concentration of 4×10^6 sperm/mL in phosphate buffered saline 1 × (PBS). Next, sperm were stained with SYBR-14 (final concentration: 32 nM) and PI (final concentration: 7.5 µM) at 38 °C in the dark for 15 min. Next, stained samples were analysed using a CytoFLEX cytometer (Beckman Coulter; Fullerton, CA, USA). SYBR-14 fluorescence was detected by the fluorescein isothiocyanate (FITC) channel (525/40), and that of PI through the PC5.5 channel (690/50). Both fluorescent probes were excited with a 488-nm laser, and no spill compensation was applied. For each sample, three technical replicates containing at least 10,000 sperm were evaluated. Throughout the entire experiment, flow rate, laser voltage and sperm concentration remained unchanged. The percentages of viable ($SYBR\text{-}14^+/PI^-$) and non-viable sperm ($SYBR\text{-}14^-/PI^+$ and $SYBR\text{-}14^+/PI^+$) were recorded and used for the subsequent statistical analysis.

Oocyte maturation, in vitro fertilisation, and embryo culture

First, ovaries from pre-pubertal gilts were collected at a local abattoir (Frigorífics Costa Brava; Riudellots de la Selva, Girona) and transported to the laboratory in 0.9% NaCl supplemented with 70 µg/mL kanamycin at 38 °C. Cumulus-oocyte complexes (COC) were retrieved from follicles and only those with complete and compact cumulus mass were selected and washed in Dulbecco's PBS (Gibco, ThermoFisher) supplemented with 4 mg/mL of BSA.

For oocyte maturation, TCM-199 (Gibco) supplemented with 0.57 mM cysteine, 0.1% (w:v) polyvinyl alcohol, 10 ng/mL human epidermal growth factor, 75 µg/mL of penicillin-G potassium, and 50 µg/mL of streptomycin sulphate was used. Groups of 40–50 COCs were transferred to a four-well multi-dish (Nunc, ThermoFisher; Waltham, MS, USA) containing 500 µL of pre-equilibrated maturation media supplemented with 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International B.V.; Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotropin (hCG; Veterin Corion; Divasa Farmavic S.A.; Gurb, Barcelona, Spain). After 20–22 h, oocytes were transferred to 500 µL of pre-equilibrated maturation media without hormones.

For the fertilisation protocol, denuded mature oocytes were placed in 50-µL drops of pre-equilibrated IVF medium containing 1 mM caffeine. The basic medium used for IVF was a modified Tris-buffered medium [69]. After adjusting semen samples to a final concentration of 1000 sperm per oocyte in IVF medium, oocytes and sperm were co-incubated for 5 h.

The presumptive zygotes were washed and transferred (40 zygotes/well) into a four-well multi-dish containing 500 µL of NCSU23 medium [70] supplemented with 0.4% BSA, 0.3 mM pyruvate and 4.5 mM lactate. After 2 days, cleaved embryos were counted to calculate the fertilisation rate; embryos were changed to NCSU23 medium supplemented with 0.4% BSA and 5.5 mM glucose, and cultured for 5 days. Embryos were classified following Balaban and Gardner [71] criteria and the percentages of morulae, early blastocysts/blastocyst, hatching/hatched blastocysts and total embryos (sum of morulae, early blastocysts/blastocyst and hatching/hatched blastocysts) were calculated on day 6 post-fertilisation. Moreover, two different ratios were determined: (i) the developmental potential of morulae on day 6, calculated as the percentage of early blastocysts/blastocysts plus hatched/hatching blastocysts divided by the percentage of morulae; and (ii) the developmental competency of fertilised embryos, calculated as the ratio between the number of embryos on day 2 and on day 6.

All procedures (oocyte maturation, IVF, and embryo culture) were carried out at 38.5 °C under a humidified atmosphere of 5% CO_2 in air. Each of the 24 ejaculates was used as a biological replicate, obtaining at least 40 zygotes per semen sample.

Neutral and alkaline Comet assays

The neutral Comet assay was used to quantify the amount of DSB, and the alkaline Comet assay was conducted to determine the whole amount of DNA breaks, including both SSB and DSB. In order to infer the amount of SSB, the neutral Comet OTM was subtracted from the alkaline Comet outcome. The protocols used for both Comet assays were previously adapted to pig sperm by Ribas-Maynou et al. [42].

Sperm fixation and lysis

First, samples were diluted to 5×10^5 sperm/mL, and mixed with low melting point agarose (37 °C) at a final concentration of 0.66%. Quickly, two drops of the mixture (6.5 µL each) were poured onto two agarose pre-treated slides, one designated for neutral Comet and the other for alkaline Comet, and covered with an 8-mm round coverslip. Thereafter, agarose was allowed to jellify at 4 °C for 5 min and coverslips were gently removed. Both slides were incubated in three lysis solutions: (1) 0.8 M Tris–HCl, 0.8 M DTT and 1% SDS for 30 min; (2) 0.8 M Tris–HCl, 0.8 M DTT and 1% SDS for 30 min; and (3) 0.4 M Tris–HCl, 0.4 M DTT, 50 mM EDTA, 2 M NaCl, 1% Tween20 and 100 µg/mL Proteinase K for 180 min.

Electrophoresis

Electrophoresis was differently conducted depending on the Comet variant. For neutral Comet, slides were electrophoresed in TBE buffer (0.445 M Tris–HCl, 0.445 M boric acid and 0.01 M EDTA; pH = 8) at 1 V/cm for 4 min, and then washed in 0.9% NaCl for 2 min. For alkaline Comet, slides were denatured in cold (4 °C) alkaline solution (0.03 M NaOH, 1 M NaCl) for 5 min, and electrophoresed in an alkaline buffer (0.03 M NaOH, pH = 13) at 1 V/cm for 4 min.

Neutralization, dehydration, and staining

Both electrophoresed slides were incubated in neutralization solution (0.4 M Tris–HCl, pH = 7.5) for 5 min, dehydrated in ethanol series (70%, 90% and 100%) for 2 min each, and allowed to dry in horizontal position. Staining was conducted using 5 µL of 1 × Safeview DNA stain (NBS biological, Huntingdon, UK), and covered with a 20 × 20 coverslip.

Imaging and analysis

An epifluorescence microscope (Zeiss Imager Z1, Carl Zeiss AG, Oberkochen, Germany) was used to observe Comets. Captures of at least 100 sperm cells per sample were conducted at 100 × magnification and resolution of 1388 × 1040 pixels, through Axiovision 4.6 software (Carl Zeiss AG, Oberkochen, Germany). Exposure time was adjusted in each capture to avoid overexposure of staining.

The quantitative analysis of the fluorescence intensity of Comet heads and tails was conducted through the open-access CometScore v2.0 software (Rexhoover, www.rexhoover.com). After automatic analysis, a manual review of each analysed Comet was conducted to remove captures not corresponding to cells, overlapping comets, or those that showed impurities that affected head or tail signal. Also, this review served to correct any inaccurate interpretation of Comet heads by the software. At this point, if the final Comet number was less than 100, more captures were performed until this figure was reached.

For the quantification of the amount of DNA breaks, OTM calculated as (Tail mean intensity − Head mean intensity) × Tail DNA/100, was chosen as a reference parameter [72].

A representative composition of images for the alkaline and neutral Comet assays, including the analysis of DNA damage conducted by the CometScore v2.0 software is shown in Fig. 3.

Statistical analysis

Data were analysed through GraphPad Prism 8.0 Software (GraphPad, San Diego, USA), and Statistics Package for Social Sciences (SPSS) ver. 25.0 (IBM Corp.; Armonk, NY, USA). For all tests, the level of significance was set as $P \leq 0.05$. First, normal distribution and homogeneity of variances were determined with Shapiro–Wilk and Levene tests, respectively. Thereafter, Spearman correlations between sperm DNA damage and sperm quality and IVF outcomes were run, and associations were assessed through multiple linear regression tests.

Subsequently, to determine the discriminant relevance of each DNA damage and sperm quality parameter for fertilisation on day 2 and total blastocyst percentage on day 6, these two IVF outcomes were divided into two groups below and above the median. A ROC analysis was used to determine the AUC of each variable, and the discriminant relevance was graded as: 0.0–0.5 no discriminant value, 0.5–0.6 failed discriminant value, 0.6–0.7 poor discriminant value, 0.7–0.8 fair discriminant value, 0.8–0.9 good discriminant value, and 0.9–1 excellent discriminant value. For all

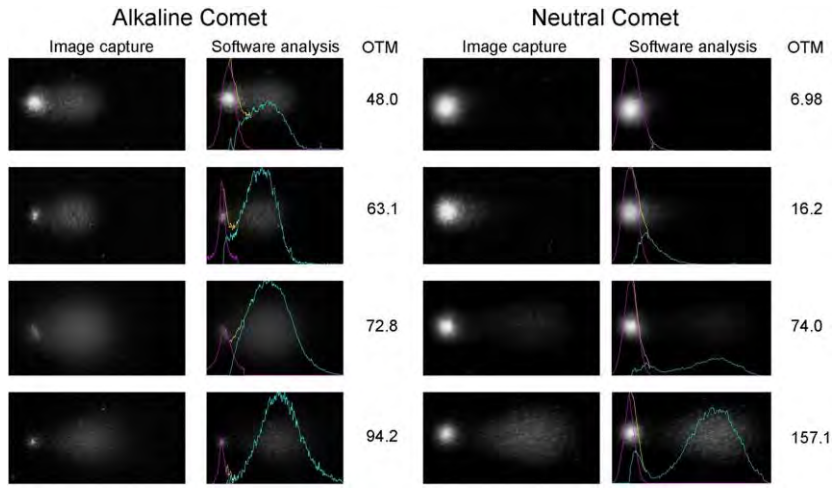

Fig. 3 Representative images for alkaline and neutral Comet assay, and their respective analysis using the Cometscore v2 software. Purple lines indicate the intensity of the comet core, blue lines indicate the intensity of the comet tail, and yellow lines indicate the superposition between core and tail. *OTM* olive tail moment

DNA damage and sperm quality parameters, sensitivity, specificity, and odds ratio were recorded.

Finally, in order to address if the addition of sperm DNA damage to the conventional semen analysis could have a higher discriminant value, a Principal Component Analysis (PCA) was generated including neutral OTM, alkaline OTM—neutral OTM, progressive motility, total motility, kinematic parameters, morphology and viability. These parameters were sorted into one PCA component, and the obtained data matrix was rotated through the Varimax procedure with Kaiser normalisation. Variables with a loading factor higher than 0.6 and lower than 0.3 in the rotated matrix were selected. The resulting coefficients were used to calculate regression scores that were assigned to each spermatozoon, and the variable was used to calculate a ROC curve for the prediction of fertilisation and blastocyst rates.

Abbreviations

ALH: Amplitude of lateral head displacement; ART: Assisted reproductive techniques; AUC: Area under the curve; BCF: Beat-cross frequency; CASA: Computer-assisted sperm analysis; COC: Cumulus-oocyte complexes; DSB: Double-strand DNA breaks; eCG: Equine chorionic gonadotropin; FITC: Fluorescein isothiocyanate; hCG: Human chorionic gonadotropin; ICSI: Intracytoplasmic sperm injection; IVF: In vitro fertilisation; LIN: Percentage of linearity; OTM: Olive tail moment; PCA: Principal component analysis; PI: Propidium iodide; ROC: Receiver operating characteristic; ROS: Reactive oxygen species; SCD: Sperm chromatin dispersion; SCSA: Sperm chromatin structure assay; SDF: Sperm DNA fragmentation; SSB: Single-strand DNA breaks; STR: Percentage of straightness; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling; VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight-line velocity; WOB: Motility parameter wobble; 8OHdG: 8-hydroxy-2′deoxyguanosine.

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Authors' contributions

Conceptualization: YM-O, JR-M and MY; methodology: YM-O, ML, AD-B, SR, EV-V and JR-M; formal analysis and investigation: YM-O, JR-M and MY; writing—original draft preparation: YM-O; writing—review and editing: IB, MY and JR-M; funding acquisition: MY; supervision: JR-M and MY. All authors have read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Since animals were not manipulated to conduct the present experiment and ejaculates were commercially acquired from a local farm (AI-centre), no permission from an Ethics Committee was required.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Publisher's Note

Discussion

Sperm have traditionally been considered as a simple vector for DNA transfer to the oocyte, capable of influencing the progeny phenotype through the delivery of their genetic material. A broader vision, nevertheless, is now accepted, as other factors have been identified as crucial for both oocyte fertilisation and embryo development. Particularly, not only has the molecular composition, primarily in terms of proteome and transcriptome, been identified as relevant (Agarwal et al., 2020; Indriastuti et al., 2022), but also DNA integrity and epigenetic marks (Erenpreiss et al., 2006; Stuppia et al., 2015; Kumaresan et al., 2020). Likewise, sperm have been reported to modulate the uterine environment, although the molecular mechanisms remain undefined (Schjenken et al., 2021). The **second Chapter of the present Dissertation, therefore, investigated the relevance of sperm molecular factors, in terms of protein and metabolite content and DNA integrity, for oocyte fertilisation and embryo development**. For this purpose, *in vitro* approaches involving oocyte IVF and IVC were carried out to address the role of these factors.

The animal model chosen for the three studies included in this Chapter was the pig. From a reproductive point of view, pigs are considered to be much closer to humans in pre-gastrulation development than other species, such as rodents (Liu et al., 2021). In particular, both humans and pigs embryos share: i) a lengthened preimplantation embryo development; ii) a flat bilaminar disc organisation rather than a cup-shaped epithelium; and iii) regulatory mechanisms for early lineage segregation, pluripotency regulation, primordial germ cell specification, and X-inactivation (Kobayashi et al., 2017; Ramos-Ibeas et al., 2019). It should be noted that, unlike humans, ungulates late blastocysts undergo an elongation process (Bolmberg et al., 2008). Considering that elongation cannot be replicated *in vitro* and that embryo development was evaluated up to hatching

blastocysts *in vitro*, this difference is irrelevant for the purpose of the studies included in this Dissertation.

The first work included in Chapter 2 intended to elucidate whether the AKR1B1 present in ejaculated sperm affected their physiology as well as fertilisation and subsequent embryo development. Despite the fact that the AKR1B1 present in SP was previously found to be positively related to *in vivo* fertility outcomes (Pérez-Patiño et al., 2018), the results from the second work of Chapter 1 showed that this relationship could not be explained by an effect on sperm function, as no relationship between SP-AKR1B1 and sperm quality and functionality parameters was observed. For this reason, the first work of Chapter 2 aimed to clarify whether SP-AKR1B1 could be transferred to sperm and, as such, could influence the reproductive success. For this purpose, three experiments were carried out: i) epididymal and ejaculated sperm AKR1B1 content was assessed using Western Blot; ii) the relationship between ejaculated sperm AKR1B1 levels and sperm quality (sperm motility, morphology and viability) and functionality (acrosome integrity, mitochondrial membrane potential and intracellular calcium) parameters was evaluated immediately after semen arrival to the laboratory and after 72 h of liquid storage; and, iii) the relationship between ejaculated sperm AKR1B1 levels and IVF outcomes was analysed.

Aldose reductase activity was previously observed in ejaculated sperm from equine (Gaitskell-Phillips et al., 2021), bovine (Frenette et al., 2003, 2004) and porcine species (Katoh et al., 2014). In the present work, a specific double-band pattern at 36 kDa and ~80 kDa in both ejaculated and epididymal sperm lysates was detected. In fact, this double-band pattern was also seen in Chapter 1, specifically in the testis, epididymis, prostate and seminal vesicles. Interestingly, this AKR1B1 double-band pattern was also identified in ovine thymus and spleen (Yang et al., 2018, 2019) and in bovine peripheral blood mononuclear cells (Yang et al., 2016). As the molecular weight of the AKR1B1 monomer is 36 kDa, the ~80 kDa band

could be a dimeric form of AKR1B1. In fact, other members of the AKR superfamily, such as xylose reductase or AKR7, need to dimerise to become active (Kavanagh et al., 2002; Kozma et al., 2002; Klimacek et al., 2003; Barski et al., 2008). This hypothesis was nevertheless discarded through denaturation experiments with urea. Thus, the ~80 kDa band could correspond to a covalent union between AKR1B1 and other molecules. In spite of this, because the two bands were confirmed to be specific after a blocking peptide assay, the following analysis envisaged 36/~80 kDa and 36 kDa/total ratios as a measurement of the putative activation state of AKR1B1.

This study also sought to investigate the potential transference of AKR1B1 from SP to sperm, as proposed in Chapter 1. This hypothesis was based on the findings reported in bovine, as : i) AKR1B1 had been described to be involved in sperm epididymal maturation (Frenette et al., 2003); and ii) AKR1B1 had been found to be contained in epididymosomes (Frenette et al., 2006). To this end, relative levels of AKR1B1 were first compared between caudal epidydimal and ejaculated sperm, but no differences were observed. These results would rule out the potential transfer of SP-AKR1B1 to sperm upon ejaculation, so the positive influence of SP-AKR1B1 on *in vivo* fertility parameters would involve other mechanisms, such as the prostaglandin synthase activity of this protein. Given its relationship with fertility, SP-AKR1B1 could enhance the PGE2 production in the uterus. Related to this, and besides its modulatory role on CL, PGE2 has been proposed to disassembly the extracellular matrix of cumulus cells, aiding sperm penetration (Niringiyumukiza et al., 2018). While this should be further analysed, the presumed enzymatic function of SP-AKR1B1 may certainly hold the key to its role on fertility.

Although the results of the second work of Chapter 1 showed that SP-AKR1B1 did not affect sperm physiology, AKR activity is understood to regulate capacitation in pigs (Katoh et al., 2014). In the present work, high

levels of the 36 kDa-band in sperm lysates were found to be strongly correlated to low intracellular calcium levels and several kinetic parameters. Calcium is involved in multiple signalling pathways like those governing sperm motility. In fact, an increase in intracellular calcium is required for mammalian sperm to switch to hyperactive movement (Ho and Suarez, 2001). This potential influence of AKR on sperm motility was previously proposed to occur not only during epididymal maturation in cattle (Frenette et al., 2003) and mice (Jagoe et al., 2013), but also during sperm capacitation in pigs (Katoh et al., 2014). In effect, AKR activity in pig was found to regulate the switch from progressive to hyperactivated movement during capacitation (Katoh et al., 2014). Although the present study did not find any relationship between the AKR1B1 contained in sperm and acrosome integrity, a potential involvement of AKR1B1 isoform during capacitation, which could entail better fertility outcomes, should be further explored under capacitating conditions.

The relationship between sperm AKR1B1 levels and *in vitro* fertilising ability was next investigated. Interestingly, the results showed that increased levels of the 36 kDa band in sperm lysates were negatively related to fertilisation rate at day 2 and to the percentage of total embryos at day 6 post-fertilisation. The explanation for such findings could reside in the activation state of the protein, mentioned above. In particular, the 36 kDa band could be considered as the inactive form of AKR1B1. Bearing in mind this possibility, it is thus reasonable to conjecture that higher levels of the active form of AKR1B1 in sperm (potentially the ~80 kDa band) could promote a switch to hyperactived motility during capacitation, which could in turn facilitate the penetration of oocyte vestments (Morales et al., 1988; Stauss et al., 1995).

All these findings would, in short, confirm the previously reported positive influence of sperm AKR1B1 on the reproductive success, acting both directly in the fertilisation process and indirectly via modulating the

uterine environment. Regarding the first, future studies should address the exact molecular mechanisms through which this would occur. For instance, as AKR1B1 is a detoxifying enzyme (Iuchi et al., 2004; Barski et al., 2008; Jagoe et al., 2013), it could exert its effect on sperm physiology through the regulation of intracellular ROS levels. Against low levels of the AKR1B1 active form, excessive ROS could cause lipid peroxidation, apoptosis-like events and DNA damage (Agarwal et al., 2008), resulting in fertilisation failure and impaired embryo development. How sperm DNA damage could influence oocyte fertilisation and embryo development in pigs is later discussed in the present Chapter. On the other hand, AKR is crucial in the polyol pathway for fructose production (Frenette et al., 2004, 2006; Jagoe et al., 2013), which could have an implication on the metabolism of pig sperm and, therefore, on their function and fertilising ability. It is, however, likely that both mechanisms are required for an optimal sperm function.

The second work included in Chapter 2 sought to determine whether sperm metabolism could affect oocyte fertilisation and preimplantation embryo development. This hypothesis arose from two facts: i) the metabolite composition of SP has been proven to influence male fertility in mammals (Mehrparavar et al., 2019); ii) the results presented in Chapter 1 indicate that energy-related metabolites, including glucose and lactate, are related to *in vivo* fertility outcomes. The repercussion of sperm bioenergetics on oocyte fertilisation and embryo development was, therefore, further investigated herein. With this aim, a targeted metabolomics approach was followed to quantify sperm intracellular metabolites related to glycolysis, ketogenesis, polycarboxylic acids cycle and oxidative phosphorylation (Oxphos) detected through LC-MS/MS. The relationship between these metabolites and sperm quality (sperm motility, morphology and viability) and functionality (acrosome integrity, mitochondrial membrane potential and intracellular calcium) parameters and IVF outcomes was then analysed.

Throughout their lifespan, not only does the metabolism of sperm adapt to the surrounding biological fluids (including SP, and uterine and oviductal fluids), but catabolic requirements also rely upon a wide range of functions, such as capacitation and oocyte penetration (Ferramosca and Zara, 2014). Consequently, sperm use different metabolic pathways, including glycolysis and Oxphos, but the preferred pathway seems to depend on the species (Storey, 2008; Rodríguez-Gil, 2013). For instance, while glycolysis appears to be the preferred energetic pathway in humans and rodents (Miki et al., 2004; Cummins, 2009), Oxphos is suggested to be predominant in horses (Moraes and Meyers, 2018), and a balance between both is seen in cattle (Losano et al., 2017). In the case of pigs, discrepancies in the metabolic pathway mainly used by sperm have been reported (Miki et al., 2004; Nesci et al., 2020), perhaps because of differences in the composition of semen preservation media. Accordingly, the second work of this Chapter first aimed to elucidate the preferred catabolic pathway used by pig sperm. The results supported that glycolysis can be regarded as the main catabolic pathway used by non-capacitated pig sperm because: i) lactate was identified in all sperm lysates; and ii) no strong positive associations between sperm physiology and Krebs cycle intermediate metabolites were detected. This finding would be supported by several additional ones. First, porcine sperm contain a specific LDH isozyme (Jones, 1997) and are able to catabolise glucose into lactate (Marin et al., 2003). Secondly, porcine sperm have mitochondria with practically invisible inner membrane crests (Rodríguez-Gil and Bonet, 2016) that, in addition, are less prominent than their horse counterparts (whose metabolism is known to be mainly oxidative) (Leung et al., 2021). Finally, the results presented in Chapter 1 suggested that higher lactate consumption is associated to higher farrowing rates. Remarkably, the energy production strategy of pig sperm resembles to that of humans (Cummins, 2009), which would make pigs a suitable animal model to further investigate the influence of metabolism on sperm physiology.

Although the sperm metabolomic profile is known to affect *in vivo* fertility outcomes in mammals (Mehrparavar et al., 2019), the exact way through which it might affect fertility is yet to be uncovered. Hence, the next step of this work was to analyse the potential influence of sperm metabolism on its function and IVF outcomes. First, the identification of a positive relationship between semen quality indicators and lactate and citrate further confirmed that sperm from high-quality samples (high sperm motility and viability, and low incidence of morphological abnormalities) preferably use glycolysis as their energy source. These results are similar to those reported in cattle (Goodson et al., 2012), mice (Miki et al., 2004; Odet et al., 2008; Danshina et al., 2010) and humans (Peterson and Fretjnd, 1970; Williams and Ford, 2001), where glycolysis was strongly related to sperm viability and motility. In addition, data also showed a positive association between Oxphos intermediates and intracellular calcium levels, which increase from the first events of capacitation (Breitbart, 2002), as detailed in Section 2.1 of the Introduction. Since capacitation should not be induced in non-capacitating extenders, high levels of intracellular calcium in sperm could be considered as an indicator of poor-quality sperm. This would further corroborate that glycolysis rather than Oxphos is related with the best sperm quality traits. On the other hand, the association between intracellular calcium levels and Oxphos metabolites might suggest a different metabolism during capacitation. In fact, a metabolism switch during capacitation has already been observed in rodents (Tourmente et al., 2022). Moreover, increased mitochondrial activity during capacitation and acrosome reaction has been observed in pigs (Ramió-Lluch et al., 2011). Yet, a characterisation of sperm metabolism under capacitating conditions should be carried out to elucidate whether this metabolic switch also occurs in pigs.

As detailed in Section 5.1.2 of the Introduction, several metabolomic studies identified specific SP and sperm metabolites as fertility biomarkers in humans (Hamamah et al., 1993; Qiao et al., 2017; Mehrparavar et al., 2019;

Mumcu et al., 2020; Xu et al., 2020), pigs (Zhang et al., 2021) and cattle (Kumar et al., 2015; Velho et al., 2018; Menezes et al., 2019; Talluri et al., 2022). The relationship between these metabolites and fertility could arise from: (i) the modulation of the maternal reproductive tract, (ii) the regulation of sperm functionality in physiological events such as capacitation, and (iii) an effect on fertilisation and embryo development. From these, only the relationship between sperm function and metabolites has been explored in humans (Wang et al., 2019), pigs (Mateo-Otero et al., 2021) and goats (Jia et al., 2021). For this reason, the second aim of this work attempted to assess the relationship between sperm metabolites, primarily those related to metabolism, and IVF outcomes.

Results showed a positive relationship between fertilisation rate evaluated at day 2 and Oxphos intermediates, and a strong positive association between the total number of embryos at day 6 and glycolysis intermediates. This could mean that, while sperm preferably using Oxphos have greater ability to fertilise an oocyte, oocytes fertilised by sperm preferably using glycolysis are able to develop up to day 6. This hypothesis would be supported by the fact that the developmental competency of fertilised oocytes was found to be positively associated to glycolysis-related metabolites. This apparent disparity between day 2 and day 6 outcomes could be explained by ROS, as sperm with high mitochondrial activity, which is directly related to Oxphos, would also have higher levels of ROS. As explained above, excessive ROS amounts are known to compromise normal sperm function through lipid peroxidation, motility reduction, apoptosis-like changes and even DNA damage (Agarwal et al., 2008). Focusing on the latter, sperm DNA damage is considered to compromise both *in vivo* and *in vitro* fertility outcomes (explained in detail in Section 5.2. of the Introduction). Thus, the data collected in the present work would indicate that excessive Oxphos activity could result in greater sperm DNA damage, which could lead to early embryonic arrest. Accordingly, oocytes fertilised by sperm using mainly glycolysis would be able to progress to late

preimplantation embryo stages. How DNA damage might be conditioning oocyte fertilisation and embryo development was addressed in the last work included in Chapter 2 and is discussed hereunder.

Sperm DNA damage has different aetiologies and may lead to different reproductive consequences in humans (Ribas-Maynou and Benet, 2019). For instance, while some authors reported a negative relationship between DNA fragmentation and fertility (Evenson and Wixon, 2006; Simon et al., 2017), others did not observe such an association (Collins et al., 2008; Zhang et al., 2015). These apparent inconsistencies have prevented from reaching a consensus on the suitability of including sperm DNA evaluation into semen routine analysis (Barratt et al., 2010; Jarow et al., 2010; Practice Committee of the American Society for Reproductive Medicine, 2015; Colpi et al., 2018; Schlegel et al., 2021). Another drawback is the absence of cut-off values for its clinical implementation, as separate techniques with different molecular basis are being currently used; the most employed methods are TUNEL, SCSA, SCF and the Comet assay (Ribas-Maynou and Benet, 2019; Agarwal et al., 2020a). While the first three are highly standardised, they cannot distinguish between single- and double-strand DNA breaks, which have been reported to have different impact on the reproductive success in humans (Agarwal et al., 2020a). Conversely, the Comet assay can be performed under alkaline or neutral pH to specifically analyse the whole amount of DNA breaks or only the double-strand ones, respectively (Ribas-Maynou and Benet, 2019).

On the other hand, in livestock, only a few authors have addressed the impact of sperm DNA breaks on fertility outcomes (Kumaresan et al., 2020). Particularly in pigs, although it has been reported that global DNA damage affects farrowing rates (Didion et al., 2009) and litter sizes (Boe-Hansen et al., 2008; Myromslien et al., 2019), no study has investigated the mechanisms underlying these observations. Bearing these considerations in mind, **the last work included in Chapter 2 intended to explore whether and**

how single- and double-strand DNA breaks influence oocyte fertilisation in pigs. To this end, sperm DNA damage was evaluated using the Comet assay, and sperm quality parameters (sperm motility, morphology and viability) and IVF outcomes were determined. In addition, the predictive value of DNA damage assessment with regard to *in vitro* fertility was also analysed through ROC analysis.

A positive relationship between the incidence of global DNA breaks and sperm motility (specifically progressive motility and several kinetic parameters) was observed in the present work. This result is in line with a previous report in pigs, in which the log-transformed DNA fragmentation index assessed by SCSA was noticed to be negatively correlated with sperm motility (Myromslien et al., 2019). This relationship was also observed in humans, as the incidence of global sperm DNA damage was determined to be negatively correlated with low sperm motility (Simon et al., 2011; Peluso et al., 2013; Belloc et al., 2014). On the other hand, the present work also found a positive relationship between global, single-strand and double-strand DNA breaks and the percentage of non-viable sperm. These results would be in accordance to those previously reported in humans, where a strong negative correlation between DNA fragmentation and sperm viability was identified (Samplaski et al., 2015). From these findings, it could be presumed that a high incidence of sperm DNA breaks occurs together with impaired sperm functionality, which could also result in decreased oocyte fertilising ability. Interestingly, data recorded in the present work did not support this hypothesis, as no relationship between DNA damage and fertilisation rate was noticed. Yet, both global and single-strand DNA breaks were established to negatively influence the number of embryos obtained at day 6. It, therefore, seems that while DNA damage does not compromise sperm fertilising competence, embryo development is impaired when levels of sperm DNA damage are high. This would be in agreement with previous studies in cattle (Fatehi et al., 2006) and humans (Tomlinson et al., 2001; Greco et al., 2005). In addition, when specific *in vitro* preimplantation

developmental stages were analysed separately, not only did global and double-strand DNA breaks negatively affect the percentage of early blastocysts/blastocysts and the percentage of hatching/hatched blastocysts, but also double-strand DNA breaks had a negative influence on the developmental potential of morulae. Similarly, an impairment of embryo developmental kinetics, reduced implantation rates and even miscarriage within the first trimester in embryos resulting from sperm containing double-strand DNA breaks were previously reported in humans, mice, cattle and goats (Ribas-Maynou et al., 2012; Gawecka et al., 2013; Scott et al., 2013; Simões et al., 2013; Casanovas et al., 2019). In fact, in agreement with the present study, single strand-breaks are not associated to embryo alterations in humans (Casanovas et al., 2019).

The early embryonic developmental arrest observed in the present work could have several explanations. First, under normal circumstances, paternal and maternal pronuclei initiate DNA replication simultaneously. In response to DNA damage, nevertheless, the male pronucleus is known to experience a delay in the onset of replication by up to 12 h, compared to the female pronucleus in mice (Gawecka et al., 2013). This non-apoptotic mechanism has also been observed to cause a delay in the progression to two-cell stage and even result in embryonic arrest before the morula stage (Gawecka et al., 2013), possibly because the degree of DNA damage exceeds the oocyte repair capacity. Another explanation would be that double-strand DNA breaks in sperm could result in chromosome aberrations, leading to both cellular apoptosis and embryo developmental arrest (Tusell et al., 1995; Alvarez et al., 1997; Scott et al., 2013). Remarkably, this would be supported by the fact that the negative repercussion of sperm DNA damage on embryo development is more obvious beyond the morula stage, as it is not until that stage that chromosome aberrations might be detected at G1/S and G2/M checkpoints (Toyoshima, 2009). This would concur with previous research reporting that chromosome aberrations

trigger morula/blastocyst arrest and apoptosis in the ICM (Adiga et al., 2007).

As already proposed by Khokhlova et al. (2020), zygotes bearing DNA damage have three main fates depending on the extent of the damage and their ability to repair it: i) mild DNA damage (possibly single-strand breaks) that can be repaired, which results in healthy offspring; ii) mild DNA damage that cannot be repaired, which potentially compromises progeny health; and iii) extensive DNA damage that cannot be repaired, which would lead to embryo failure to develop and implant (Khokhlova et al., 2020). Yet, the molecular mechanisms underlying DNA damage repair are less investigated in embryos compared to other cell types. Elucidating these mechanisms would thus help to better understand how embryonic arrest is induced.

Finally, this work confirmed that the inclusion of the Comet assay into the conventional semen quality assessment could improve the prediction of blastocyst development at day 6, but not that of cleavage rates at day 2. While this is the first report quantifying the benefit of including the Comet assay in the routine semen analysis in farm animals, previous studies in pigs (Boe-Hansen et al., 2005; Didion et al., 2009; Myromslien et al., 2019) and cattle (Januskauskas et al., 2001; Karoui et al., 2012; Dogan et al., 2015; Kumaresan et al., 2017) already traced the clinical significance of other sperm DNA fragmentation assays. Thus, the present work joins the advice already given in a recent study in humans indicating that routine testing of sperm DNA integrity improves the prediction of ART success (Ribas-Maynou et al., 2021).

In summary, the present Chapter showed that the sperm AKR1B1 activation state, sperm metabolism and sperm DNA integrity directly or indirectly impact oocyte fertilisation and, even, embryo development (**Figure 6**). In more detail, the effect of both AKR1B1 and sperm metabolism on fertility was postulated to be explained by ROS production. ROS have

been demonstrated to directly impair embryo genome activation, which causes developmental arrest and, eventually, cell death (Deluao et al., 2022). Yet, another indirect mechanism by which ROS could affect embryo development would be the induction of DNA damage (Bui et al., 2018). Further studies should thus clarify whether low levels of AKR1B1 and/or Oxphos are related to high ROS levels, and which their direct and indirect implications on embryo development are.

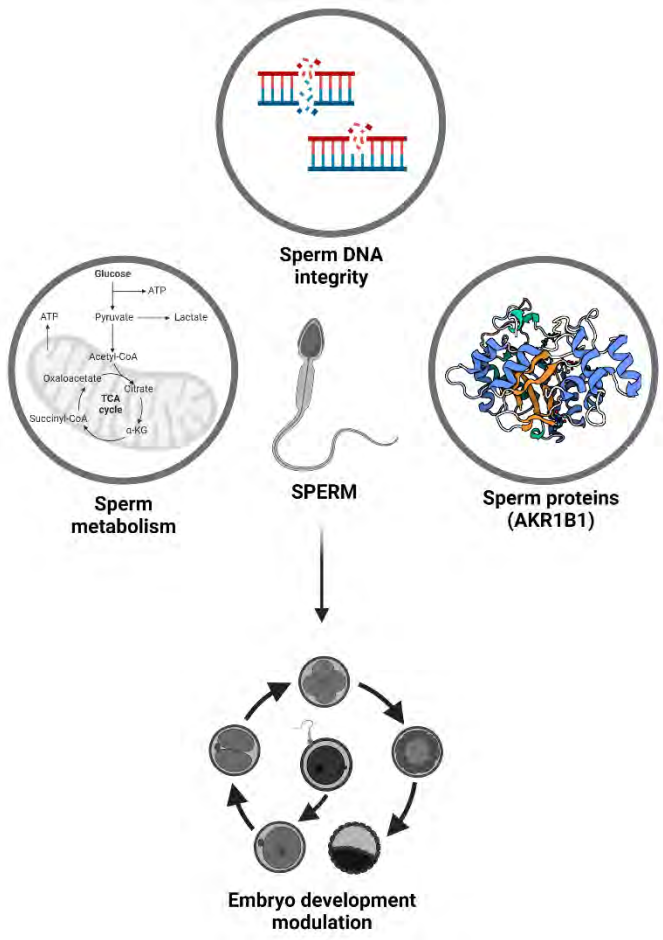

Figure 6. **Sperm factors influence on *in vitro* fertility outcomes.** Schematic representation of the main findings of the present Chapter. Created with BioRender.

General Discussion

Mammalian reproductive biology has traditionally been focused on the study of gamete interaction. Progressively, many additional factors have been established to affect the reproductive success, involving both the female and male sides. Consequently, in the last decades, several efforts have been made towards the identification and quantification of their specific impact on fertility.

Although paternal factors have been demonstrated to have a direct influence on reproductive physiology and progeny in mammalian species (Parinaud et al., 1993; Bromfield et al., 2014; Morgan et al., 2019), the underlying molecular mechanisms by which these components can shape the process have not been fully characterised. For many years, the paternal genome carried by male gametes was purported to explain this influence. In the last decades, however, different studies have reported a biological contribution of seminal factors further beyond the sperm genome. In particular, mounting evidence supports the idea that the male contribution to fertility success is determined by seminal factors carried by both SP and sperm (Colaco and Sakkas, 2018; Vallet-Buisan et al., 2023). Indeed, seminal factors seem to be able to modulate a wide range of reproductive process, involving those related to both male and female physiology, which ultimately have an impact on fertilisation and embryo development (Schjenken and Robertson, 2020). Because the molecular mechanisms underlying this contribution are largely unknown, the overall objective of the present Dissertation was to elucidate the contribution of SP and sperm to reproductive outcomes, using cattle and pigs as animal models.

Seminal factors have been reported to be able not only to affect sperm functioning but also to interact and regulate the female reproductive tract. Focusing on the latter, seminal factors are known to: i) trigger the uterine inflammatory response in mice (Robertson et al., 1996; Tremellen et al., 1998), humans (Sharkey et al., 2012b, 2012a), cows (Ibrahim et al., 2019), horses (Troedsson et al., 2001), sheep (Scott et al., 2006) and pigs (O'Leary

et al., 2006); ii) modulate the ovarian function in induced (Adams and Ratto, 2013) and non-induced (Gangnuss et al., 2004; O'Leary et al., 2006; Bogle et al., 2011; Tanco et al., 2012) ovulatory species; and iii) elicit the secretion of embryotropic factors by the uterus and oviduct in mice (Robertson et al., 1992; Bromfield et al., 2014), pigs (O'Leary et al., 2004) and cattle (Loureiro et al., 2009). Taking all these aspects into account, the present Dissertation firstly investigated whether uterine priming with SP regulates the development of *in vitro* produced embryos in cattle (**Paper I**). The data presented here demonstrated that uterine exposure to SP is able to improve embryo development, in line with the results observed in other species such as pigs (Martinez et al., 2019), mice (Bromfield et al., 2014) and humans (Tremellen et al., 2000). Several explanations for the exact mechanisms by which SP positively influences embryo development have been proposed. Focusing on cattle, SP has been found to induce the expression of endometrial inflammatory mediators (Ibrahim et al., 2019), which would result in leukocytic recruitment to prepare the uterine environment for a successful implantation and to further support pregnancy. In addition, the secretion of embryotropic factors by the endometrium in response to seminal factors (Schjenken and Robertson, 2020) could also explain the beneficial effect of SP on embryo development. On the other hand, although SP from induced ovulatory species has been found to modulate cattle ovarian physiology (Tanco et al., 2012), this did not seem to occur in this work. The absence of differences in the CL size between groups suggests that bovine SP does not modulate ovarian function, in a similar fashion to that observed in mice (Gangnuss et al., 2004). It is noteworthy that the present Dissertation identified a potentially new SP modulatory capacity, particularly through the synthesis of specific PG by the AKR1B1 present in SP. The lack of relationship between SP-AKR1B1 and sperm function parameters, together with the fact that the highest levels of the protein were found in SRF-P2 and post-SRF (**Paper II**) indicate that the positive influence of this protein on fertility (Pérez-Patiño et al., 2018) would be related to its

effects on the female reproductive tract. Interestingly, the AKR1B1 secreted by the endometrium was previously reported to act as a prostaglandin synthase in humans (Bresson et al., 2011), cattle (Madore et al., 2003) and pigs (Seo et al., 2014), influencing the production of pro-luteolytic and pro-luteal factors PGF2α and PGE2, respectively (Bresson et al., 2012). It is therefore plausible that the AKR1B1 present in SP joins uterine AKR1B1 upon ejaculation, potentially enhancing PGE2 production for improved fertility outcomes. Yet, as no increase in CL size was observed in the study carried out in bovine (**Paper I**) after either 7 or 14 days of SP deposition, how AKR1B1 modulates fertility in cattle should be analysed in further studies. In any case, these findings indicate that SP-molecules could be able to join female enzymes to enhance the production of several modulatory molecules, which would be likely to regulate the uterine environment and even oocyte fertilisation and embryo development. This should be further investigated to achieve a more comprehensive perspective of the influence of SP on fertility.

As briefly mentioned above, the modulation of fertility by seminal factors could also entail sperm physiology regulation. In this Dissertation, the effect of molecules contained in both sperm and SP on sperm function was investigated by analysing proteins, particularly AKR1B1 (**Paper II** and **IV**), and metabolites (**Paper III** and **V**). Regarding the former, the AKR1B1 contained in SP was seen to not influence sperm function (**Paper II**), suggesting that it could affect fertility outcomes through other mechanisms. For instance, one could hypothesise that the positive influence of SP-AKR1B1 on fertility outcomes would first imply a transference to sperm upon ejaculation. In spite of this, no differences in AKR1B1 levels were observed between epididymal and ejaculated sperm (**Paper IV**). Collectively, the results from these two works suggest that the positive impact of SP-AKR1B1 on fertility relies upon the modulation of the female reproductive tract rather than sperm physiology. Additionally, this Dissertation subsequently evaluated the link between the activation state of AKR1B1 and

sperm functionality and fertilisation ability (**Paper IV**). Interestingly, a relationship between sperm AKR1B1 levels and *in vitro* oocyte fertilisation and embryo development in pigs was found. One could posit two explanations for such observations. First, the AKR1B1 present in ejaculated sperm could be able to modulate sperm fertilising ability, probably via regulating sperm capacitation (Katoh et al., 2014). Yet, considering AKR1B1 has been described to act as a detoxifying enzyme via the reduction of carbonyl-containing metabolic compounds (Iuchi et al., 2004; Barski et al., 2008; Jagoe et al., 2013), another possibility could be that higher AKR1B1 activation leads to lower ROS levels.

The results from the untargeted metabolomic analysis of SP suggested that SP metabolites might influence *in vivo* fertility outcomes probably through the modulation of multiple reproductive events (**Paper III**). In effect, metabolites related not only to specific traits of sperm physiology, such as motility, capacitation or acrosome integrity, but also to cell metabolism, were identified as potential *in vivo* fertility biomarkers. Focusing on the latter, as reduced levels of glucose and lactate in SP were found to be positively associated to better reproductive outcomes, the involvement of sperm metabolism on the reproductive success was, therefore, further explored in this Dissertation. Data suggested that sperm metabolism influences fertility outcomes in terms of both *in vitro* oocyte fertilisation and early embryo development (**Paper V**). In particular, sperm that preferably use glycolysis as the main catabolic pathway were noticed to produce more embryos at later developmental stages. Although an explanation for these findings would be related to the modulation of sperm functioning, another possibility would entail oxidative stress, as higher mitochondrial activity would result in increased ROS production.

Excessive ROS are known to compromise normal sperm function through lipid peroxidation, motility reduction, apoptosis-like changes and even DNA damage (Agarwal et al., 2008). From these insults, sperm DNA

damage has been observed to compromise fertility in mammalian species (Kumaresan et al., 2020; Ribas-Maynou et al., 2021). The last work of this Dissertation (**Paper VI**) thus aimed to analyse how single- and double-strand DNA breaks affect porcine fertility, specifically oocyte fertilisation and embryo development. Similar to cattle (Fatehi et al., 2006) and humans (Tomlinson et al., 2001; Greco et al., 2005), data included here demonstrated that, while sperm DNA damage does not impair sperm fertilising ability, it compromises embryo development in pigs. In particular, double-strand breaks seemed to have a dramatic impact on the percentage of early and late blastocysts, indicating that unresolved DNA damage is likely to trigger early embryo arrest. These results would explain previous observations in porcine, where sperm DNA damage was noticed to negatively affect litter size (Boe-Hansen et al., 2008; Myromslien et al., 2019). Even though some mechanisms, such as the oocyte incapability to repair extensive DNA damage (Gawecka et al., 2013) and chromosomal aberrations (Tusell et al., 1995; Alvarez et al., 1997; Scott et al., 2013), have been proposed to explain embryonic arrest, how these mechanisms may exactly undermine embryo developmental potential remains to be clarified. In addition, further research should address whether the activation state of AKR1B1 and/or sperm metabolism are related to ROS levels and sperm DNA damage in order to fully explain the observed relationship with fertility outcomes in both cases.

Conclusions

The main conclusions of this Dissertation are:

1 Uterine exposure to seminal plasma improves *in vivo* preimplantation embryo development, but does not increase embryo survival rates in cattle. Although the mechanisms underlying this improvement remain unknown, they do not seem to involve ovarian regulation.

2 The AKR1B1 is expressed in all the organs of the boar reproductive tract, except bulbourethral glands. In addition, the levels of AKR1B1 in seminal plasma do not seem to significantly influence sperm quality and functionality.

3 Specific metabolites present in pig seminal plasma, such as lactate, carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate, glucose, citrate, creatine, malonate, phenylalanine, tyrosine and fumarate, are related to *in vivo* fertility outcomes in pigs. All these metabolites are thus suggested as biomarkers to predict *in vivo* fertility.

4 AKR1B1 in pig sperm modulates their motility, probably via regulating calcium homeostasis. In addition, the activation state of AKR1B1 in sperm is related to their fertilising ability and the *in vitro* development of embryos they give rise to.

5 Sperm basal metabolic activity determines *in vitro* fertility outcomes in pigs. Particularly, glycolysis rather than Oxphos is used by sperm as the main energetic pathway in good quality samples. Additionally, glycolysis seems to be the preferred catabolic pathway in sperm leading to greater *in vitro* embryo development rates.

6 Whilst, in pigs, sperm DNA damage does not affect *in vitro* fertilisation outcomes, it induces embryonic arrest at early developmental stages.

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