Universitat Autònoma de Barcelona

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UNDER THE SUPERVISION OF:

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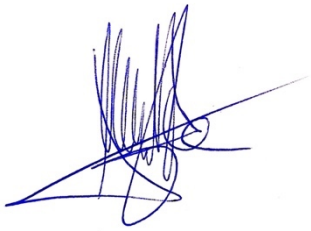

Dr. Manuel Álvarez Rodríguez

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(Doctorando)

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A mi familia,
qué suerte compartir con vosotros
el amor por la biología.

A esos faros,
por iluminar en la calma
por guiar en la tempestad.
Gracias.

« …Qualunque cosa farai, amala,
come amavi […],
quando eri picciriddu ».

Cinema Paradiso, 1988

No hay camino,
sino estelas,
en la mar.

a Papá

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

A	Adenine
ABHD2	Alpha/beta hydrolase domain-containing protein 2
Ab-Am	Antibiotic-Antimycotic
ACTB	Beta-actin
ACTH	Adrenocorticotrophic hormone
AI	Artificial insemination
Amp	Ampulla
ANOVA	Analysis of variance
ANXA1	Annexin A1
AR	Androgen receptor
ARA	Arachidonic acid
ART	Assisted reproduction technique
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
β-ACTIN	Beta-actin
β-NGF	Beta-nerve growth factor
BAX	BCL2 associated X apoptosis regulator
BCL2	BCL2 apoptosis regulator B-Cell leukemia/lymphoma 2
BP	Biological process
bp	Base pair
BSA	Bovine serum albumin
BTS	Beltsville thawing solution
C	Cytosine
Cas9	CRISPR-associated protein 9
CatSper	Cation channel of sperm
cDNA	Complementary deoxyribonucleic acid
COC	Cumulus-oocyte complex
COQ6	Ubiquinone biosynthesis monooxygenase COQ6
CoQ10	Coenzyme Q10

COX1	Prostaglandin-endoperoxide synthase 1
COX2	Prostaglandin-endoperoxide synthase 2
CO_2	Carbon dioxide
CR	Corticosteroid receptor
CRH	Corticotropin-releasing hormone
CRISPR	Clustered regularly interspaced short palindromic repeats
CV	Coefficient of variation
Cvx	Cervix
$C_{59}H_{90}O_4$	Coenzyme Q10
DAPI	4′,6-Diamidino-2-phenylindole
DBD	DNA binding domain
DC	Detergent compatible
DEG	Differentially-expressed gene
DistUt	Distal uterus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia* (for example)
EG	Ethylene glycol
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ERα	Nuclear receptor subfamily 3 group A member 1 Estrogen receptor alpha
ERβ	Nuclear receptor subfamily 3 group A member 2 Estrogen receptor beta
ESR1	Estrogen receptor alpha gene
ESR2	Estrogen receptor beta gene
et al	*Et alii*; *et aliae* (and others)
E2	Estradiol
F	Forward
FAO	Food and Agriculture Organization of the United Nations

FBS	Fetal bovine serum
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FKBP	FK506-binding peptidyl-prolyl cis/trans isomerase protein
FKBP4	FK506-binding peptidyl-prolyl cis/trans isomerase protein 4
	FK506-binding peptidyl-prolyl cis/trans isomerase protein 52
FKBP5	FK506-binding peptidyl-prolyl cis/trans isomerase protein 5
	FK506-binding peptidyl-prolyl cis/trans isomerase protein 51
FKBP51	FK506-binding peptidyl-prolyl cis/trans isomerase protein 5
	FK506-binding peptidyl-prolyl cis/trans isomerase protein 51
FKBP52	FK506-binding peptidyl-prolyl cis/trans isomerase protein 4
	FK506-binding peptidyl-prolyl cis/trans isomerase protein 52
G	Guanine
GAG	Glycosaminoglycans
GC	Glucocorticoid
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GR	Nuclear receptor subfamily 3 group C member 1
	Glucocorticoid receptor
GRE	Glucocorticoid response element
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HSD11B	11-beta-Hydroxysteroid dehydrogenase
HSD11B1	Hydroxysteroid 11-beta dehydrogenase 1
HSD11B2	Hydroxysteroid 11-beta dehydrogenase 2
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSPA4	Heat shock protein family A (HSP70) member 4
HSPA4L	Heat shock protein family A (HSP70) member 4-like

HSPA5	Heat shock protein family A (HSP70) member 5
HSPA8	Heat shock protein family A (HSP70) member 8
HSP90AB1	Heat shock protein 90 alpha family class B member 1
i.e.	*Id est* (that is)
IGFBP1	Insulin-like growth factor binding protein 1
IL-6	Interleukin 6
IL-10	Interleukin 10
im	Intramuscular
Inf	Infundibulum
Isth	Isthmus
IVF	*In vitro* maturation
IVM	*In vitro* fertilization
JAK	Janus kinase
KEGG	Kyoto encyclopedia of genes and genomes
LBD	Ligand binding domain
LCA	*Lens culinaris* agglutinin
LDS	Lithium dodecyl sulfate
LH	Luteinizing hormone
LME	Linear mixed effects
LN_2	Liquid nitrogen
MED1	Mediator complex subunit 1
MED14	Mediator complex subunit 14
MM	Maturation media
mo	Months old
MR	Nuclear receptor subfamily 3 group C member 2 Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MII	Metaphase II
NaCl	Sodium chloride
$Na_2C_6H_6O_7$	Sodium hydrogen citrate
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NR	Nuclear receptor
NR3A1	Nuclear receptor subfamily 3 group A member 1 Estrogen receptor alpha
NR3A2	Nuclear receptor subfamily 3 group A member 2 Estrogen receptor beta
NR3C1	Nuclear receptor subfamily 3 group C member 1 Glucocorticoid receptor
NR3C2	Nuclear receptor subfamily 3 group C member 2 Mineralocorticoid receptor
NR3C3	Nuclear receptor subfamily 3 group C member 3 Progesterone receptor
NR3C4	Nuclear receptor subfamily 3 group C member 4 Androgen receptor
NTD	N-terminal domain
OCM	Oocyte culture medium
OVGP1	Oviductal glycoprotein 1
PANTHER	Protein analysis through evolutionary relationship
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PC1	First principal component
PC2	Second principal component
PDB	Protein Data Bank
PDSS2	Decaprenyl diphosphate synthase subunit 2
PGH2	Prostaglandin H2
PGRMC1	Membrane-associated progesterone receptor component 1
PGRMC2	Membrane-associated progesterone receptor component 2
PLA2G4B	Phospholipase A2 group IV B
PR	Nuclear receptor subfamily 3 group C member 3 Progesterone receptor
ProxUt	Proximal uterus
PTGS1	Prostaglandin-endoperoxide synthase 1

PTGS2	Prostaglandin-endoperoxide synthase 2
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus
P4	Progesterone
Q8	Coenzyme Q8
Q10	Coenzyme Q10
qPCR	Quantitative real-time polymerase chain reaction
R	Reverse
RFU	Relative fluorescence units
RIN	Ribonucleic acid integrity values
RIPA	Radio-immunoprecipitation assay
RMA	Robust multi-array average
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Real Time
RU486	Mifepristone
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SP	Seminal plasma
ST	Stage
STAT	Signal transducers and activators of transcription
STAT1	Signal transducers and activators of transcription 1
STAT2	Signal transducers and activators of transcription 2
STAT3	Signal transducers and activators of transcription 3
STAT5A	Signal transducers and activators of transcription 5A
STAT5B	Signal transducers and activators of transcription 5B
STAT6	Signal transducers and activators of transcription 6
SUMO	Small ubiquitin-like modifier
T	Thymine
TAC	Transcriptome analysis console

TBP	TATA-box binding protein
Tm	Melting temperature
TLR2	Toll-like receptor 2
TP53	Tumor protein P53
UTJ	Utero-tubal junction
UV	Ultraviolet
VS	Vitrification solution
ZP	Zona pellucida
11β-HSD	11-beta-Hydroxysteroid dehydrogenase

SUMMARY

The assisted reproduction techniques have greatly improved over the last decades. However, there is still a long way to go until acceptable success rates are achieved. Developing strategies for reducing the cellular stress *in vitro*, together with a better understanding of signaling pathways involved in reproductive physiology and fertility, can provide new valuable insights in the field. The regulation of glucocorticoids (GCs) can be a candidate signaling pathway in this regard. GCs are steroid hormones that can inhibit reproduction during stress responses, but are also essential at baseline levels for important events of the female reproductive physiology, including ovulation, oocyte maturation or implantation. The role of GCs in reproduction is complex and seems to be different among species. The mechanisms involved in their regulation remain unclear, but most of their actions are attributed to the GC receptor, NR3C1, to the enzymes controlling GC availability, and immunophilins FKBP4 and FKBP5, involved in the regulation of the NR3C1 actions in the nucleus. Accordingly, the present thesis studies the role of the gene and protein expression of the GC receptor –and molecules involved in GC regulation– in the reproductive function, by determining its modulation in the female reproductive tract during reproductive events *in vivo* and *in vitro*. In this regard, we studied the GC regulatory expression in bovine oocytes and cumulus during *in vitro* maturation, vitrification, and supplementation with coenzyme Q10 (Q10), an endogenous molecule with important mitochondrial properties. Thus, we demonstrated that Q10 is beneficial for preserving the oocyte integrity after vitrification, exerts positive effects against apoptosis, and can influence the regulation of GCs in the female gamete. Moreover, we determined the changes in GC-related expression occurring at baseline level in the bovine endometrium

and ampulla across different stages of the estrous cycle. These changes showed a spatiotemporal pattern that matched with reproductive events, suggesting a relevant role of GCs in the oviduct during the postovulatory phase. After this approach, we aimed to understand the influence of the male-female interaction in the expression of the GC regulation, by assessing the effects of natural mating, artificial insemination and seminal plasma infusion in the reproductive tract of preovulatory sows. According to our results, the natural mating induces a tight regulation for the restriction of the GC actions in the sperm reservoir which is not mimicked by artificial insemination. Finally, in the rabbit, an induced-ovulation species, we evaluated the modulation of *NR3C1* in the female reproductive tract caused by the influence of the male-female interactions, and during different time points, corresponding to reproductive events such as ovulation, fertilization and presumed embryo developmental stages. We concluded that seminal plasma could trigger *NR3C1* expression in the infundibulum, and mating increased *NR3C1* in a spatiotemporal sequence corresponding to the assumed location of rabbit embryos. Overall, this thesis provides new knowledge about the complex GC regulation in reproduction and its modulation during reproductive events in different species. A better comprehension of this pathway may help to unravel the underlying mechanisms behind the stress influence on reproduction, find novel fertility biomarkers, and develop new potential strategies with reproductive purposes.

RESUMEN

Las técnicas de reproducción asistida han mejorado enormemente durante las últimas décadas. Sin embargo, todavía existe un largo que camino que recorrer hasta alcanzar tasas de éxito que sean aceptables. El desarrollo de estrategias para mitigar el estrés celular *in vitro*, junto con una mayor comprensión de rutas de señalización implicadas en la fisiología reproductiva y la fertilidad, puede proporcionar nuevas valiosas perspectivas para el ámbito. La regulación de los glucocorticoides (GCs) puede ser una ruta de señalización candidata en este sentido. Los GCs son hormonas esteroideas que pueden inhibir la reproducción durante las respuestas a estrés, pero que son esenciales a nivel basal para importantes eventos de la fisiología reproductiva en la hembra, incluida la ovulación, la maduración ovocitaria o la implantación. El papel de los GCs en la reproducción es complejo y parece variar en función de la especie. El mecanismo involucrado en su regulación no está claro por el momento, pero muchas de sus acciones se atribuyen al receptor de GCs, NR3C1, y también a las enzimas que controlan la disponibilidad de GCs, así como a las inmunofilinas FKBP4 y FKBP5, implicadas en la regulación de las acciones de NR3C1 en el núcleo. En consecuencia, en la presente tesis se estudia el papel de la expresión génica y proteica del receptor (y otras moléculas implicadas en la regulación de GCs) en la función reproductiva, mediante la determinación de su modulación en el tracto reproductivo de la hembra durante diferentes eventos reproductivos *in vivo* e *in vitro*. En este sentido, se estudió la expresión de la regulación de GCs en células del cúmulus y ovocitos de bovino sujetos a maduración *in vitro*, vitrificación y suplementación con coenzima Q10 (Q10), una molécula endógena con importantes propiedades a nivel mitocondrial. Así, se demostró que la Q10 es beneficiosa para la preservación de la integridad del ovocito post-vitrificación,

que ejerce un efecto positivo contra la apoptosis, y que puede influir en la regulación de los GCs en el gameto femenino. Además, se determinaron los cambios en la expresión basal relacionada con los GCs en el endometrio y la ampolla del bovino, a largo de las diferentes fases del ciclo estral. Estos cambios mostraron un patrón espaciotemporal de coincidencia con los eventos reproductivos, sugiriendo una función relevante de los GCs en el oviducto durante la fase postovulatoria. Tras este enfoque, el objetivo fue entender la influencia de la interacción macho-hembra en la expresión de la regulación de GCs, evaluando los efectos de la monta natural, la inseminación artificial y la infusión con plasma seminal en el tracto reproductivo de cerdas en fase preovulatoria. De acuerdo a nuestros resultados, la monta natural induce una regulación muy controlada para la restricción de la acción de los GCs en el reservorio espermático, la cual no sucede tras la inseminación artificial. Finalmente, en conejo, especie de ovulación inducida, se evaluó la modulación de *NR3C1* en el tracto reproductivo después de la interacción macho-hembra, y durante diferentes puntos temporales, que se corresponden con eventos reproductivos como la ovulación, la fecundación y los estadios embrionarios. Se concluyó que el plasma seminal puede inducir la expresión de *NR3C1* en el infundíbulo, y que la monta natural aumenta *NR3C1* en un patrón espaciotemporal coincidente con el de los embriones. En conjunto, esta tesis aporta nueva información sobre la compleja regulación de los GCs en la reproducción, y su modulación durante los eventos reproductivos en diferentes especies. Una mayor comprensión de esta ruta puede ayudar a descifrar los mecanismos subyacentes a la influencia del estrés en la reproducción, encontrar nuevos biomarcadores de fertilidad, así como desarrollar nuevas estrategias con fines reproductivos.

RESUM

Les tècniques de reproducció assistida han millorat enormement durant les darreres dècades. No obstant això, encara hi ha un llarg camí a recórrer per tal d'assolir taxes d'èxit que siguin acceptables. El desenvolupament d'estratègies per mitigar l'estrès cel·lular *in vitro*, juntament amb una millor comprensió de rutes de senyalització implicades en la fisiologia reproductiva i la fertilitat, pot proporcionar noves valuoses perspectives per aquest àmbit. La regulació dels glucocorticoides (GCs) pot ser una ruta de senyalització candidata en aquest sentit. Els GCs són hormones esteroidals que poden inhibir la reproducció durant les respostes al estrès, però que són essencials a nivell basal per a importants esdeveniments de la fisiologia reproductiva a la femella, incloent l'ovulació, la maduració del oòcit o la implantació. El rol dels GCs a la reproducció és complex i sembla variar en funció de l'espècie. El mecanisme involucrat en la seva regulació no és clar actualment, però moltes de les seves accions s'atribueixen al receptor de GCs, NR3C1, i també als enzims que controlen la disponibilitat de GC, així com a les immunofilines FKBP4 i FKBP5, implicades en la regulació de les accions de NR3C1 al nucli. En conseqüència, en aquesta tesi s'estudia el rol de l'expressió gènica i proteica del receptor (i altres molècules implicades en la regulació de GCs) en la funció reproductiva, mitjançant la determinació de la modulació en el tracte reproductiu de la femella durant diferents esdeveniments reproductius *in vivo* i *in vitro*. En aquest sentit, es va estudiar l'expressió de la regulació de GCs en cèl·lules del cúmulus i oòcits de boví sotmesos a maduració *in vitro*, vitrificació i suplementació amb coenzim Q10 (Q10), una molècula endògena amb importants propietats a nivell mitocondrial. Així, es va demostrar que la Q10 és beneficiosa per a la preservació de la integritat de l'oòcit post-vitrificació, que exerceix un efecte positiu contra l'apoptosi, i que pot influir en la regulació dels

GCs en el gàmeta femení. A més, es van determinar els canvis en l'expressió basal relacionada amb els GCs a l'endometri i l'ampolla del boví, al llarg de les diferents fases del cicle estral. Aquests canvis van mostrar un patró espai-temporal de coincidència amb els esdeveniments reproductius, suggerint una funció rellevant dels GCs a l'oviducte durant la fase postovulatòria. Després d'aquesta aproximació, l'objectiu va ser entendre la influència de la interacció mascle-femella en l'expressió de la regulació de GCs, avaluant els efectes de la munta natural, la inseminació artificial i la infusió amb plasma seminal al tracte reproductiu de truges en fase preovulatòria. D'acord amb els nostres resultats, la munta natural indueix una regulació molt controlada cap a la restricció de l'acció dels GCs al reservori espermàtic, la qual cossa no succeeix després de la inseminació artificial. Finalment, en el conill, espècie d'ovulació induïda, es va avaluar la modulació de *NR3C1* al tracte reproductiu causada per la influència de la interacció mascle-femella, així com durant diferents punts temporals, que es corresponen amb esdeveniments reproductius, com l'ovulació, la fecundació i els estadis embrionaris. Es va concloure que el plasma seminal pot induir l'expressió de *NR3C1* a l'infundíbul, i que la munta natural augmenta *NR3C1* en un patró espai-temporal coincident amb el dels embrions. En conjunt, aquesta tesi aporta nova informació sobre la complexa regulació dels GCs a la reproducció, i la seva modulació durant els esdeveniments reproductius en diferents espècies. Una millor comprensió d'aquesta ruta pot ajudar a desxifrar els mecanismes subjacents a la influència de l'estrès en la reproducció, trobar nous biomarcadors de fertilitat, així com desenvolupar noves estratègies amb finalitats reproductives.

INTRODUCTION

Humanity is –and will relentlessly continue– facing great challenges regarding sustainable development issues. Some of those are related to climate change, which is currently causing detrimental effects on worldwide food supplies (Godber & Wall, 2014; Ray *et al.*, 2019), spread of infectious diseases (Butler *et al.*, 2021), and loss of biodiversity (Turney *et al.*, 2020). In this sense, it is of great interest the conservation of diverse animal breeds that may be potentially helpful for adapting to the changing environmental conditions, and increasing the efficiency in food provision (Henry *et al.*, 2018). Animal genetic resources could be used for achieving reproductive success in different species by using assisted reproductive techniques (ARTs), including cryopreservation of germplasm (gametes and embryos), artificial insemination, *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* embryo culture and embryo transfer. All these techniques have greatly improved in the last decades, however there is still a long way to go in research and development until acceptable success rates of reproductive performance are achieved (Hansen, 2014; Presicce, 2020).

Regarding cryopreservation, gametes and embryos have been preserved using cryoprotectants and slow freezing methods for years, achieving different results depending on the species (Argyle *et al.*, 2016). Nevertheless, the oocyte, the largest mammalian cell, has remained the most difficult to cryopreserve as a result of its low surface area to volume ratio, which favors the creation of intracellular ice crystals that can damage the plasmatic membrane (Tharasanit & Thuwanut, 2021), combined with a low permeability to water and cryoprotectants (Leibo, 1980). These difficulties have encouraged the replacement of the slow freezing technique by the vitrification method, which allows the solidification of the cell with the aid of extremely viscous cryoprotectants and fast cooling rates, avoiding the formation of intracellular ice and minimizing the membrane chilling injury. Moreover, vitrification presents additional convenient advantages, being

cheaper, less time-consuming and requiring less equipment (Mogas, 2019). In the recent years, the vitrification of bovine oocytes has become of particular interest for the storage of female genetic resources either for conservation or commercial purposes (Andrabi & Maxwell, 2007; Silva *et al.*, 2015). However, in the bovine species, the rates of successful IVF and embryo development of vitrified oocytes still remain low and there is a need to improve them (Hwang & Hochi, 2014). There are several plausible explanations for these suboptimal outcomes, although damage during the oocyte IVM and cryodamage during vitrification are believed to be important contributing factors for these results (Combelles *et al.*, 2009; Saragusty & Arav, 2011).

In this context, different strategies have been develop with the aim of achieving better outcomes, including methodological approaches and the supplementation with antioxidants during the IVM prior to vitrification (Iwata, 2021; Mogas, 2019). Antioxidant supplementation can be helpful to mitigate some the specific damages that can be caused by IVM and cryopreservation, including damage to the membranes, altered meiotic spindle, anomalies in the cortical granules distribution, DNA damage, ATP depletion, alteration of the mitochondrial function, oxidative stress, including the increased presence of reactive oxygen species, or apoptosis (Combelles *et al.*, 2009; Gupta *et al.*, 2010; Len *et al.*, 2019). Coenzyme Q10 (Q10) is an endogenous antioxidant present in the inner mitochondrial membrane that has been proposed for this approach due to its putative roles in the mitochondrial function (Ben-Meir *et al.*, 2015), oocyte reduction of aneuploidies (Ma *et al.*, 2020), cellular redox balance (Quinzii *et al.*, 2010), apoptosis (Zhang *et al.*, 2019), and gene transcription (Gendelman & Roth, 2012). In ***Chapter I*** of this thesis, the potential effects of Q10 supplementation during IVM of bovine fresh and vitrified oocytes are studied on survival, meiotic spindle conformation, and cortical granules migration. In ***Chapter II***, the changes

in the mRNA expression of genes related to apoptosis and the regulation pathway of glucocorticoids (GCs) are studied after IVM, Q10 supplementation, and vitrification of bovine oocytes and cumulus cells.

Despite the evidences of the role of GCs in gene transcription, energy or metabolism in reproductive events (Wang & Harris, 2015; Whirledge & Cidlowski, 2017), their specific functions and mechanisms in oocyte maturation and female reproductive physiology have remained controversial and unclear, showing considerable differences across species (Gong *et al.*, 2017; González *et al.*, 2010; Scarlet *et al.*, 2017; Simmons *et al.*, 2010; Whirledge *et al.*, 2015; Yang *et al.*, 1999). In this sense, it seems crucial to unravel the gap in knowledge of the GC function in reproductive physiology, which may allow a better understanding the actual implications of GCs in this area.

The GCs are steroid hormones that display pleiotropic roles on diverse crucial functions of the mammalian physiology, including energy metabolism (Vegiopoulos & Herzig, 2007), growth (Swarbrick *et al.*, 2021), inflammation (Cain & Cidlowski, 2017), cognitive function (Mizoguchi *et al.*, 2004), homeostasis (Hunter *et al.*, 2014), circadian rhythms (Dickmeis, 2009), cardiovascular function (van der Sluis & Hoekstra, 2020), development (Wyrwoll, 2014), stress response (Geraghty & Kaufer, 2015), and also, reproduction (Whirledge & Cidlowski, 2017). The HPA axis activation during the stress response can disrupt the reproductive function at different levels and GCs are well recognized for their effects inhibiting reproduction, associated to acute stress, or causing infertility when chronic stress induces sustained pathological levels. Nevertheless, GCs are also essential for the correct reproductive function in the female, playing roles in reproductive events such as follicle maturation, ovulation, luteinization, fertilization, immune regulation, implantation and pregnancy (Acosta *et al.*, 2005; da Costa *et al.*, 2016;

Tetsuka, 2007; Tetsuka & Tanakadate, 2019; Whirledge & Cidlowski, 2013; Yding Andersen, 2002). Are then GCs good or bad for reproduction? Probably both. It could be easily argued that *high levels* are detrimental. However, what are *high levels*? The HPA axis varies taxonomically and the differences in circulating GCs between species can be huge, ranging from a 1-3 ng/mL in the lemon shark (*Negaprion brevirostris*) or the Magellanic penguin (*Spheniscus magellanicus*) to 2000-4000 ng/mL in New World monkeys, such as the marmoset (*Callithrix jacchus*) (Romero, 2004). Non-stress baseline circulating GC levels of one species may be more than sufficient to cause death in another species, which does not mean that some species are more stressed than others. These differences are not only found in absolute GC levels, but also in the impact that an increase of GC exposure may have on the physiological functions, including reproduction, of the different species (Fanson & Parrott, 2015). In other words, some species may be more "sensitive" to the detrimental effects of GCs and high levels may be relatively low for them (Gong *et al.*, 2017; Whirledge & Cidlowski, 2013; Yang *et al.*, 1999). However, the origin of those great variations across taxa and their evolutive significance (if any) still remain unknown (Romero & Gormally, 2019). Actually, the diversity of the GC effects is even more complex, as GC regulation is determined in a tissue-specific manner, meaning that the different tissues do not exert a common response to the same GC signal (Lattin *et al.*, 2015; Martins & de Castro, 2021), which raises questions about their expression in the tissues of the female reproductive tract. Regarding these issues, GC-related expression studied on this thesis is analyzed in several tissues of the female reproductive tract of different species, including bovine (***Chapter III***), porcine (***Chapter IV***) and rabbit (***Chapter V***). In addition, variations in the GC-related expression can also present differences during the stages of the reproductive cycle, which is the central focus in bovine tissues in ***Chapter III***.

At this point, it is appropriate to highlight that although hormone exposure is important, they are mere chemical messengers. Beyond GC release, their main functional relevance is related to the effects they induce downstream (Romero & Gormally, 2019). As aforementioned, GCs are essential but can also be detrimental for reproductive physiology and their biological effects can cause pathology or, by contrast, mediate adequate physiological functions. To this duality could be applied the Aristotelian philosophical concept of *aurea mediocritas*, or *golden mean*, which is not related to mediocrity but to the desirable balance between the two extremes, one of excess and the other of deficiency (Aristotle, 1955). This *balance* in actual GC exposure and the extent of the GC actions in the cells requires of a tight regulation that involves a great number of enzymes and co-factors (Timmermans *et al.*, 2019). As a result of their lipophilic characteristics, free GCs can diffuse through the plasma membrane of the cells. However, the effective bioavailability of GCs in the cell cytoplasm is actually controlled by two enzymes, the 11β-hydroxysteroid dehydrogenases (HSD11Bs), which catalyze the conversion of inactive cortisone (11-dehydrocorticosterone in rodents) into active cortisol (corticosterone in rodents) (HSD11B1), and the opposite reaction (HSD11B2). Thus, the expression levels of these enzymes, regulate the GC exposure in the different tissues or cells of the different species, acting as gatekeepers (Chapman *et al.*, 2013). Then, many of the GC effects are mediated by their homonym receptor, NR3C1 (or GR) (Rhen & Cidlowski, 2005; Timmermans *et al.*, 2019). Upon GC binding, it is translocated to the nucleus where it acts as a transcription factor, regulating gene transcription of great number of pathways. The action of the receptor in the nucleus involves the formation of a complex, including FKBP4 and FKBP5, which seem to be relevant in the regulation of the GC functions, promoting or hindering the translocation of this complex (Ratajczak *et al.*, 2015).

Identifying protein-coding genes with relevance in the reproductive events of each species seems to be one of the key next steps in research in the field of reproduction (Schimenti & Handel, 2018). The expression of the biological mechanisms related to the fine-tuning of the complex GC pathway in reproduction, including the NR3C1 receptor, but also other regulatory *checkpoints*, such as HSD11B1, HSD11B2, FKBP4, FKBP5 and other factors, are the *leitmotif* throughout this document. Their roles in the reproductive function, both in the oocyte and the female reproductive tract, have remained *terra incognita*. It is the aim of this thesis to shed some light on the complexity of this matter that, if completely understood, may potentially revert to future applications in ARTs. Thus, in ***Chapter IV*** are mapped the GC-related transcriptomic changes occurring in the different tissues of the porcine reproductive tract in response to mating, sperm-peak fraction artificial insemination, or only its seminal plasma. In ***Chapter V***, the *NR3C1* mRNA expression is studied in the different tissues along the rabbit reproductive tract in response to seminal plasma and mating. Moreover, the influence of the latest is evaluated at different time points, corresponding to specific reproductive events, such as ovulation, fertilization, and different stages of the rabbit early embryo development.

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LITERATURE REVIEW

GLOBAL CHALLENGES: WORLD POPULATION, CLIMATE CHANGE & BIODIVERSITY CONSERVATION

According to the projections of United Nations, the current global population (7.8 billion), will grow to 9.7 billion by 2050 (United Nations, 2019). The constantly raising population rates have increased the demand of food supplies, threatening food systems and global nutrition, but also sustainable development of ecosystems, agriculture and livestock production. Current food systems entail a risk for future nutrition due their negative impact on climate change through greenhouse gas emissions, desertification, water use, overuse of chemical inputs, biodiversity loss, and emergence of zoonotic diseases caused by the destruction of ecosystems (United Nations, 2021). In this sense, conservation of the biodiversity of animal species and breeds is a precious resource for adapting to the changing environmental conditions, and increasing the efficiency of food production for reaching global sustainable development goals (FAO, 2012). In 1868, Charles Darwin wrote: "*wonderful skill and perseverance shown by the men who have left an enduring monument of their success in the present state of our domesticated animals*" (Darwin, 1868). However, nowadays, around 26% of the world livestock breeds (~7000) are at risk of extinction, and 83% of the wild cattle native species of North America, Eurasia and Africa are threatened, according to the first report on global assessment of biodiversity for food and agriculture, *The State of the World's Biodiversity for Food and Agriculture* (FAO, 2019). In this report, it is also highlighted the added value of preserving many species and breeds that may have particular traits that help them to cope with the climate change effects (e.g., higher temperatures, lower rainfall, greater pressure from diseases…), potentially increasing the resilience of food production, and subsequently, the capability of facing global challenges. As an example, criollo

livestock breeds in Panama or Mexico show higher fertility rates, increased resistance to diseases, and can eat poor quality pastures in arid and semiarid environments (Anderson *et al.*, 2015; FAO, 2019).

CONSERVATION OF ANIMAL GENETIC RESOURCES

The development and implementation of conservation plans should consider the conservation of animal genetic resources as a priority, according to the *Global Plan of Action for Animal Genetic Resources Conservation and the Interlaken Declaration* (FAO, 2012). There are many reasons for the conservation of these resources, including economic reasons, related to animal production; socio-cultural reasons, related to the traditional cultural roles played by some livestock and wild species in the identity particular human communities around the world; environmental reasons, both in developing and developed countries; reduction of genetic risks by maintaining the diversity of breeds and species; and research purposes, including basic research in reproduction, genetics, and adaptation to climate change, among others (FAO, 2012). The conservation of animal genetic resources can be addressed differently, depending on the pursued objectives.

IN VIVO STRATEGIES

The conservation of *in vivo* species or breeds involve the maintenance of live animal populations, which can be *in situ* or *ex situ* (FAO, 2012). For *in situ* conservation, i.e., involving conservation in the same environment/community where the breed evolved or is normally found, it is required to induce a change in the local economy and market dynamics. The allowance of economic viability of the breed in the community is crucial for the maintenance of the breed genetic

diversity, due to the risk of genetic loss caused by the presence of other breeds that may be commercially predominant. *Ex situ in vivo* conservation involves preservation of live animals outside of the environment/community where they were evolved or are normally found. This conservation normally involves controlled conditions outside of the original environment and a small number of animals. It can be beneficial for the conservation of critically endangered species due to the higher possibility of breeding activity in a controlled environment and the absence of predatory pressure. However, it is strongly recommended to use this strategy only for a short period before reintroduction in the native habitats, owing to animal welfare and stress concerns. Moreover, *ex situ in vivo* approaches should be used always in combination with i*n vitro* strategies (FAO, 2012).

IN VITRO STRATEGIES

In vitro conservation of animal genetic resources is not based in the preservation of live animals or populations, but in the creation of gene bank collections of cryopreserved genetic material, such as tissue fragments, or germplasm that can be preserved long term (FAO, 2012). The main objectives of these repositories are achieving animal reproductive success by the use of assisted reproductive techniques (ARTs), besides allowing the advancement, step by step, on the knowledge on these mentioned fields thanks to basic and applied research efforts. The creation of gene banks supports the conservation *in situ*, and allows the long term safeguard of genetic material of wild species and rare, endangered or native breeds of farm animals. It is a powerful tool, being and insurance of the biodiversity for the future generations that may inherit those resources. Potentially, germplasm conservation may allow the recreation of extinct species or breeds. Moreover, it allows the transport of germplasm of specific breeds

between gene banks, facilitating conservation, commercial interests and scientific research projects. Therefore, *in vitro* conservation strategies require technical knowledge in reproductive physiology, genetics and also cryobiology.

CRYOPRESERVATION: THE COLDEST ART

Cryopreservation is a technique developed for the preservation of biological resources at extremely low temperatures, normally in liquid nitrogen (-196°C). This approach relies on the idea of "stopping" cellular metabolism, which could be preserved for incredibly long periods of time at those temperatures, before being "restarted" after thawing (Mazur, 1963). The vitrification of germplasm constitutes a very useful application of this technology, and it is of great interest for optimizing the protocols.

CRYOPROTECTIVE AGENTS

The major difficulty of cryopreservation relies on avoid damages (cryoinjury) to the cells during the process, including intracellular ice formation, which can mechanically break the plasmatic membranes and induce detrimental effects related to the low temperatures. To overcome these effects, appropriate cooling/thawing rates and the use of cryoprotectant agents have been applied. Different types of cryoprotectants have been used for this purpose, including cell membrane permeable (such as dimethyl sulfoxide (DMSO), glycerol, ethylene glycol and propanediol) and non-permeable agents (such as polyvinyl pyrrolidone, trehalose, raffinose, and other sugars) (Whaley *et al.*, 2021). The use of cryoprotectants is applied for the two cryopreservation approaches more used nowadays for germplasm cryopreservation, slow freezing and vitrification.

Permeable cryoprotectants enter the cells and create hydrogen bonds with water molecules, depressing the water freezing point and avoiding the creation of nucleation sites, required for crystal formation. At specific ranges of concentration, some permeable cryoprotectants, such as DMSO, increase cellular permeability, favoring the replacement of water by cryoprotectants. On the other hand, non-permeable cryoprotectants exert their action from outside, dehydrating the cell by osmosis caused by higher solute extracellular concentrations. Although both types of cryoprotectants can induce toxic effects to the cells at high concentrations, non-permeable are less harmful (Pegg, 2007).

CRYOPRESERVATION TECHNIQUES FOR GERMPLASM

During slow freezing the cells are gradually exposed to a low concentration of cryoprotectants, and equilibrated at temperatures from -5°C to -7°C for several minutes. After, seeding is initiated and extracellular freezing is induced at a slow cooling rate of 0.3-0.5°C/min, causing a gradual intracellular dehydration, until a temperature range between -30°C and -65°C is reached, and then directly plunged in liquid nitrogen (Mazur, 1963; Saragusty & Arav, 2011). In contrast, vitrification involves the creation of a "glassy" amorphous state that acts as a solid during an ultra-rapid cooling in liquid nitrogen. This state can be achieved due to a high intracellular viscosity provided by cryoprotectants, which difficult the formation of ice crystals. However, a high viscosity state requires a higher concentration of cryoprotectants that may rise concerns regarding cellular toxicity. In this sense, minimizing the sample volumes of concentrated cryoprotectants, and achieving fast cooling (and warming) rates are key factors for success of vitrification (Vajta & Kuwayama, 2006). Slow cooling has been successfully applied in the cryopreservation of germplasm of different species for many years

(Saragusty & Arav, 2011). However, vitrification provides greater advantages in terms of success rates, together with cheaper costs, less time needed, and simpler equipment required, which may ease the straightforward cryopreservation of wild species in field conditions (Argyle *et al.*, 2016).

Even when vitrification is currently the most used cryopreservation technique for reproductive purposes, the success rates for oocyte vitrification still need to be improved. The oocyte has remained especially difficult to vitrify successfully in part due to a low surface-to-volume ratio and a low permeability to cryoprotectants and water (Leibo, 1980; Tharasanit & Thuwanut, 2021). Moreover, the reproductive diversity present across species often results in particularities in their germplasm that require specific protocols and techniques that are not yet tuned for some species (Comizzoli *et al.*, 2019). Despite the great commercial interest of cattle, the vitrification of bovine oocytes has remained considerably limited and the embryo developmental rates from vitrified oocytes are much lower than those derived from slow freezing (Hwang & Hochi, 2014). In this sense, experimental research for developing strategies focused on improving the bovine oocyte quality *in vitro* seems essential for achieving better outcomes.

OOCYTE STRESS *IN VITRO*

Cryopreservation and *in vitro* techniques have improved over the last decades. However, *ex vivo* management of oocytes unfailingly requires exposition to conditions different than those found *in vivo*, involving incubation in artificial conditions and synthetic mediums. Despite the efforts to mimic the natural environment, or cause less cryoinjury (in the case of cryopreservation), oocyte vitrification (Len *et al.*, 2019; Nohales-Córcoles *et al.*, 2016), *in vitro* maturation (IVM) (Combelles *et al.*, 2009) and *in vitro* embryo culture (Hardy *et al.*, 2021;

Takahashi, 2012) are still stressful events that can impair ARTs success and fertility outcomes.

IN VITRO MATURATION

Regarding oocyte maturation, there are a great number of studies reporting differences between *in vivo* and *in vitro* matured oocytes, in terms of metabolism, spindle formation and gene expression (Li *et al.*, 2006; Lonergan *et al.*, 2003; Sanfins *et al.*, 2003; Sutton *et al.*, 2003). In addition, cytoplasm maturation may be hampered compared to nuclear maturation, creating asynchrony in the maturation process (Ali *et al.*, 2006; Gilchrist & Thompson, 2007; Trounson *et al.*, 2001). Also, embryos derived from IVM show a higher number of abnormalities (Nogueira *et al.*, 2000; Schramm *et al.*, 2003). In this sense, the generation of oxidative stress *in vitro* when the oocyte is still maturing may be playing an important role in the outcomes of reproductive success (Combelles *et al.*, 2009).

CRYODAMAGE

The IVM is often followed by oocyte vitrification, which is a stressful process for the cells. We already mentioned the risks of ice formation during the process, which are diminished using cryoprotectants that at the same time, may cause osmotic damage and toxicity to the cells. Moreover, *in vitro* matured oocytes at metaphase II are considerably more sensitive to cryoinjury compared to oocytes at germinal vesicle (Tucker *et al.*, 1998), as a result of the spindle formation, the peripheral distribution of cortical granules and a low permeability, or compared to embryo blastomeres, due to differences in the surface-to-volume ratio and the composition of the plasma membrane (Kawai *et al.*, 2011). Thus, vitrification may

have a great impact on the oocyte, causing premature cortical granules exocytosis, depolymerization and disorganization of microtubules and damage to the meiotic spindle (Hwang & Hochi, 2014). Oocyte membranes could also be easily damaged by the osmotic pressure caused during the shrinkage and permeation of cryoprotectants, since the actin filaments of the oocyte membrane are less resistant (Mogas, 2019). In the oocytes of some large domestic species, such as the bovine, the high lipidic content of the cytoplasm may also be an important factor in cryoinjury, increasing the oocyte sensitivity to low temperatures (Arav *et al.*, 1996).

Also, cryopreservation seems to induce the creation of elevated levels of reactive oxygen species (ROS) (Len *et al.*, 2019). Multiple mechanisms may be associated to this increase, including alterations in the mitochondrial membrane potential during the chilling process (Jones *et al.*, 2004). Oxidative stress and redox unbalance caused by vitrification can induce several pernicious effects to the oocytes, including damage to the mitochondrial membrane, low electron density of the mitochondrial matrix, ATP depletion, altered mitochondrial morphology and localization, DNA fragmentation, altered signaling and also, apoptosis (Agarwal *et al.*, 2005; Iwata, 2021). The main factors that may challenge the oocyte are shown in **Figure 1**.

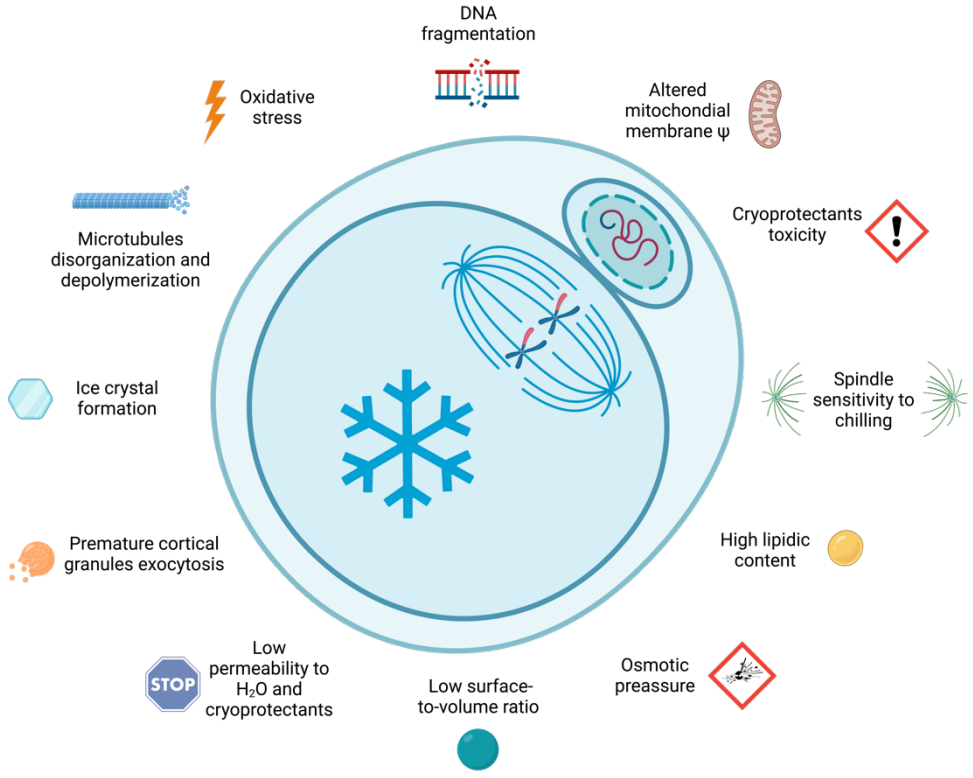

Figure 1. Graphical representation of the main factors that can affect the mammalian oocytes during cryopreservation procedures.

STRATEGIES TO MITIGATE OOCYTE DAMAGE

In this context, different strategies have been applied during the last years for mitigating the oocyte damage caused by *ex vivo* procedures and improve the reproductive outcomes, including the increase of permeability to water and cryoprotectants by the expression of aquaporins (Edashige *et al.*, 2003); the partial removal of cytoplasmic lipid droplets (Prates *et al.*, 2014); the mild exposure to sublethal stressors (Pribenszky *et al.*, 2010); and the coincubation with substances that may help the oocytes to overcome the stressful impact of *in vitro* procedures and cryopreservation techniques. Thus, molecules with antioxidant properties may be beneficial for mitigating the high damage associated to the increase in

ROS and mitochondrial impairment. Resveratrol (Iwata, 2021; Piras *et al.*, 2019), melatonin (Qin *et al.*, 2021; Soto-Heras *et al.*, 2019), glutathione ethyl-ester (García-Martínez *et al.*, 2020), alpha-tocopherol (Yashiro *et al.*, 2015), or L-carnitine (Sprícigo *et al.*, 2017) are some examples of substances that have been reported to have positive effects on the IVM and vitrification of oocytes and embryos of domestic and laboratory species. Since mitochondria is at the same time the main source of ROS and one of the most sensitive organelles to oxidative stress, mitochondria- targeted antioxidants may be of great interest for mitigating the vitrification damage (Gualtieri *et al.*, 2021). In this sense, coenzyme Q10 (Q10) could be a potential candidate to mitigate the oocyte damage caused by vitrification.

COENZYME Q10 IN REPRODUCTION

Coenzyme Q10 (CoQ10 or Q10) is also known as ubiquinone as a result of its ubiquity in the mitochondria and the lipid membranes (Stockwell, 2019). Its molecular structure is depicted in **Figure 2**. It is an essential component of the mitochondrial electron transport chain of the inner membrane of the mitochondria, acting as an electron carrier in the complex I and III, preventing electron leakage to oxygen and consequently ROS production (Hernández-Camacho *et al.*, 2018). Q10 is also crucial for ATP production inside mitochondria, an indispensable antioxidant in extramitochondrial membranes, and essential in the synthesis of pyrimidines (López-Lluch *et al.*, 2010).

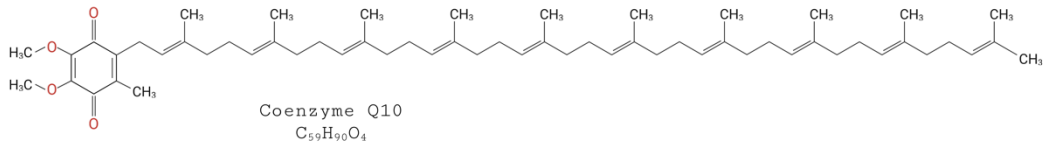

Figure 2. Coenzyme Q10 (Q10) chemical structure. It is an endogenous molecule synthesized in animal cells composed of a benzoquinone ring and a polyisoprenoid tail of ten subunits. The tail has a variable length depending on the species. While yeasts have six subunits (Q6), *Escherichia coli* has eight (Q8) and rodents nine (Q9). Humans and most mammals have ten subunits (Q10)

Since the mitochondria of the embryo are maternally inherited, the oocyte mitochondrial function may be crucial for the embryo development. In this sense, oocytes with greater ATP levels (at least 2 pmol/oocyte) showed better development and implantation (Van-Blerkom *et al.*, 1995). Regarding the Q10 influence in female infertility, *PDSS2* and *COQ6* genes of Q10 synthesis were found downregulated in old age oocytes (Ben-Meir *et al.*, 2015a), and ATP depletion and mitochondrial decreased activity were associated with Q10 deficiency (Ben-Meir *et al.*, 2015b). Moreover, increased levels of Q10 in follicular fluid have been associated with oocyte maturation, optimal embryo morphokinetic parameters and pregnancy rates (Akarsu *et al.*, 2017; Turi *et al.*, 2012). Further, Q10 supplementation during IVM increased the maturation rates of human oocytes and reduced the presence of post-meiotic aneuploidies (Ma *et al.*, 2020). Besides, Q10 can also have beneficial effects on the regulation of gene expression (Xing *et al.*, 2021). In bovine, Q10 supplementation during IVM improved embryonic development, mitochondrial features and mitigated the effects of thermal stress (Gendelman & Roth, 2012; Stojkovic *et al.*, 1999).

Overall, Q10 seems to play an important role in the mitigation of oocyte aging (Ben-Meir *et al.*, 2019; Bentov & Casper, 2013; Meldrum *et al.*, 2016), which

is greatly induced by oxidative stress. In this regard, Q10 may act scavenging the increase in ROS, alleviating mitochondrial dysfunction, reducing DNA damage and controlling the activation of apoptosis (Zhang *et al.*, 2019). For these reasons, Q10 seems a promising candidate to alleviate the detrimental effects of oocyte vitrification and stress *in vitro*.

GENERAL PHYSIOLOGY OF GLUCOCORTICOIDS

Throughout evolution, multicellular organisms have acquired multiple traits that allow them to adapt in a continuously changing environment. In order to maintain homeostasis, different biological mechanisms have evolved to provide a physiological adaptation in response to potential life-threatening situations, such as infection, food deprivation or stress (Granner *et al.*, 2015). Steroid hormones (e.g., cortisol, testosterone, estradiol, progesterone, corticosterone, aldosterone), are non-polar molecules consisting of three 6-carbon rings and a conjugated 5-carbon ring, which act as key messengers in some of these essential processes, mediating in the signaling between the stimulus and the response. Every signal needs a receiver, and steroid receptors are the lock for the steroid hormones key (Kushiro *et al.*, 2003). As steroid hormones are lipid-soluble, they are able to cross plasma membranes, binding to receptors allocated either in the nucleus, or the cytoplasm. Even though free steroids can exert a signal on their own, most of their functions are mediated by binding to steroid receptors. Thus, the hormone-receptor complex travels to the nucleus, where binds to hormone-response-elements in the DNA, acting as a transcription factor and triggering the increase and/or decrease in the expression of specific genes (Garland *et al.*, 2016).

Glucocorticoids (GCs) are steroid hormones that are vital for the mammalian physiology (Timmermans *et al.*, 2019). They are named because of

their main role in the regulation of the glucose metabolism (Vegiopoulos & Herzig, 2007), but they are also essential in a kaleidoscope of functions (Buckingham, 2006), having pleiotropic effects on cognition (Mizoguchi *et al.*, 2004), the immune response (Cain & Cidlowski, 2017), the cardiovascular function (Cruz-Topete *et al.*, 2016), the electrolyte balance (Hunter *et al.*, 2014), growth (Mazziotti & Giustina, 2013), reproduction (Whirledge & Cidlowski, 2017) and fetal development (Moisiadis & Matthews, 2014), and many more physiological functions. In mammals, these hormones are synthesized in the mitochondria of the zona fasciculata, the middle zone of the adrenal gland cortex (Bose *et al.*, 2002; Midzak & Papadopoulos, 2016). In addition, extra-adrenal production of GCs has been also observed in some tissues, including the intestine, the brain, the lungs, the placenta and the skin (Ahmed *et al.*, 2019; Phan *et al.*, 2019; Slominski *et al.*, 2020). These non-adrenally produced GCs are thought to have a more local (and specific) impact in physiology, and may also be independent of the circadian rhythms. The classical biosynthesis pathway of GCs is shown in Figure 3. Cholesterol is the common precursor of all the different GCs. While corticosterone is the dominant GC in most reptiles, amphibians, birds (Koren *et al.*, 2012), cortisol is the main GC in most fish and mammals (Carbajal *et al.*, 2019; Sheriff *et al.*, 2011), with the exception of rodents, where corticosterone is the major GC (Palme, 2019). In lagomorphs, corticosterone has been described as the predominant GC in the literature, however, there is no consensus in this regard. In this sense, differences in the major GC secreted have been reported under different stress conditions, and among different lagomorph species (Hamilton & Weeks Jr, 1985).

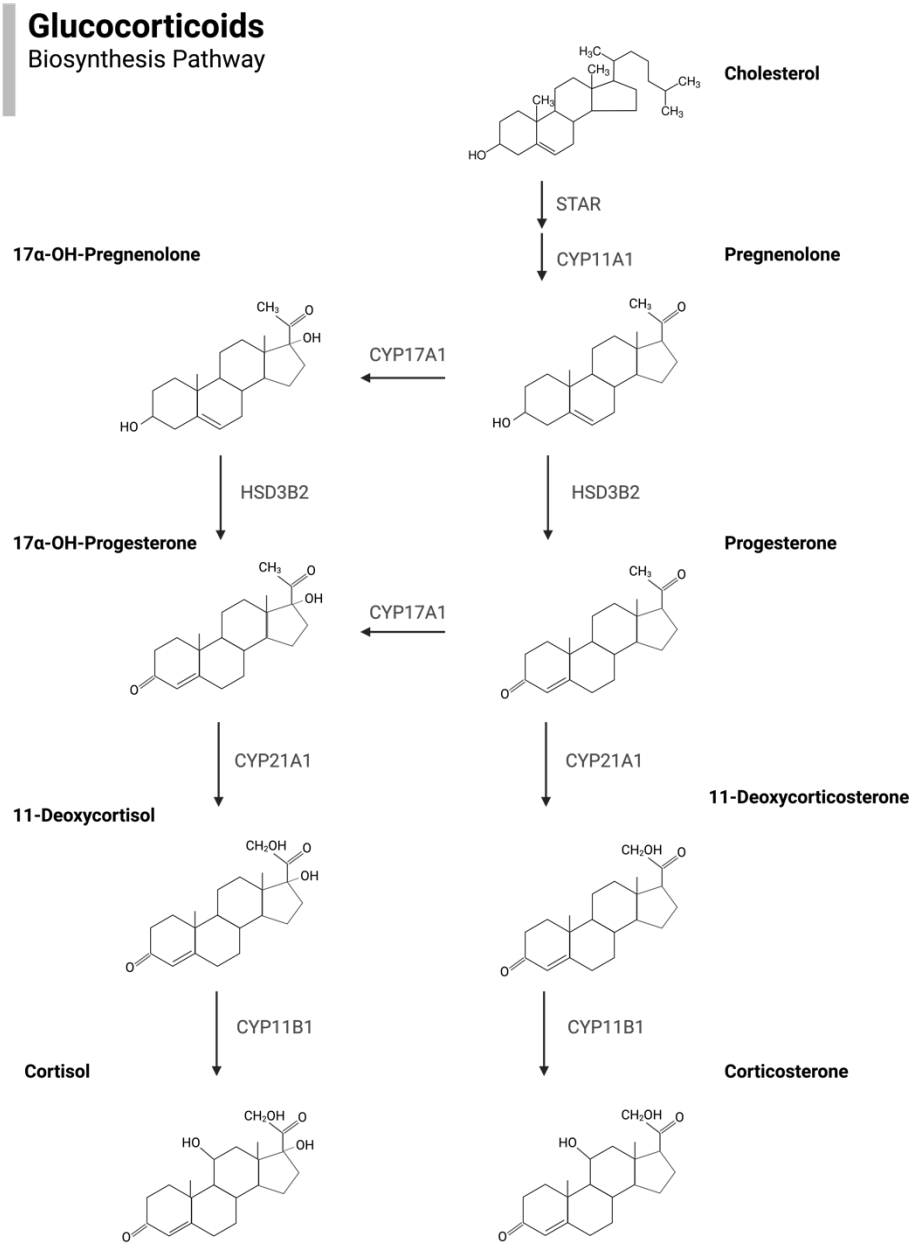

Figure 3. Biosynthesis pathway of the most predominant glucocorticoid (GC) in non-rodent (Cortisol) and rodent mammals (Corticosterone), synthesized from cholesterol in the adrenal glands. The different conversion steps until bioactive GCs are synthesized are catalyzed by different enzymes, shown in the figure. The 17-α hydroxylase (CYP17A1) is present in most mammals, but absent in rodents.

The hypothalamic-pituitary-adrenal axis (HPA) is the main regulator of the systemic GC production and release. The paraventricular nucleus (PVN) neurons of the hypothalamus release corticotropin-releasing hormone (CRH) and also vasopressin (AVP), that reach the anterior lobe of the pituitary gland subsequently inducing the secretion of adrenocorticotrophic hormone (ACTH). Then, circulating ACTH binds to cognate receptor in the adrenal gland, stimulating the biosynthesis and release of GCs directly into the bloodstream (Sacta *et al.*, 2016).

Circulating GC levels are influenced with circadian rhythms and ultradian oscillations. These patterns are controlled by the suprachiasmatic nucleus in response to day and light periods that in turn switch the HPA axis (Dickmeis, 2009). Moreover, the GC activity is regulated by emotional and physical stressful stimulus. Specific stress inputs from the amygdala, the hippocampus, the prefrontal cortex, the locus coeruleus and the bed nucleus of the stria terminalis are driving the HPA axis stimulation (Arango-Lievano *et al.*, 2015). The pathway of the HPA activation of until GCs release is shown in Figure 4.

While acute GC release is a "positive counter-response" for the animals to cope with stressors, chronic or sustained GC secretion has been traditionally related to pernicious effects for the body. However, the total amount of GCs in the blood variates very much among species, as some have physiological circulating levels that may be well above and beyond all bearing for other species (Romero & Gormally, 2019). Even more, the GC release of some species seems to be attenuated to the point that there is no change in their levels despite being exposed to stressful situations, and to show great seasonal variations (Romero, 2002). Furthermore, the impact of stress seems to be very disparate among the different body tissues (Lattin *et al.*, 2015). All these evidences may point to a dynamic equilibrium in the GC response to cope with the stress, which is not very

conserved between species, even if they are phylogenetically close (Dickens & Romero, 2013; Romero & Gormally, 2019). This variability in terms of function, makes a problematic issue establishing a profile of GC release associated to stress from time point GC measures in blood (Schoenemann & Bonier, 2018). Thereon, it may be potentially more effective and biologically relevant to examine the downstream steps of the GC signaling.

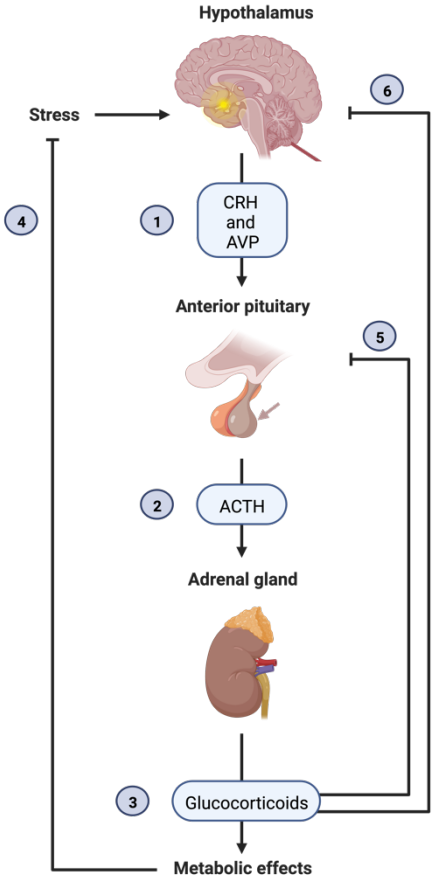

Figure 4. HPA response triggered during stress. An stressful stimulus triggers the secretion of CRH by the neurons in the PVN of the hypothalamus (**1**). Then, CRH and AVP induce the secretion of ACTH in the anterior pituitary, traveling *via* the systemic circulation to the adrenal cortex (**2**). In the adrenal cortex, ACTH activates the synthesis of GCs (**3**). Upon release, GCs trigger metabolic effects towards the alleviation of the effects of stress, including anti-inflammatory effects and gluconeogenesis (**4**). The GCs, exert a negative feedback, inhibiting the secretion of ACTH in the pituitary (**5**) and CRH and AVP in the hypothalamus (**6**).

LOCAL AVAILABILITY OF GLUCOCORTICOIDS

The exposure to GCs in a specific tissue is mainly controlled by the action of two enzymes, the HSD11Bs, 11β-HSD1 which acts predominantly as an NADP(H)-dependent reductase, generating active cortisol (or corticosterone in rodents), and 11β-HSD2, which is a high affinity NAD^+-dependent enzyme that catalyzes the inactivation of GCs (Michael *et al.*, 2003). The enzymatic transformation of GCs are shown in **Figure 5**. These enzymes are responsible for the GC availability to the GC receptor, and seem to be directly implicated in regulating their actions in reproduction, such as ovulation and oocyte maturation and fertilization (Anbo *et al.*, 2022; Gong *et al.*, 2017; Geraghty & Kaufer, 2015). However, while their main functions activating or inactivating GCs are clear, great differences regarding the expression of these enzymes have been found between species, and their role regarding oocyte sensitivity to GCs have remained controversial for years (da Costa *et al.*, 2016; Gong *et al.*, 2017; Scarlet *et al.*, 2017; Tetsuka *et al.*, 2003; Tetsuka & Tanakadate, 2019; Yang *et al.*, 1999).

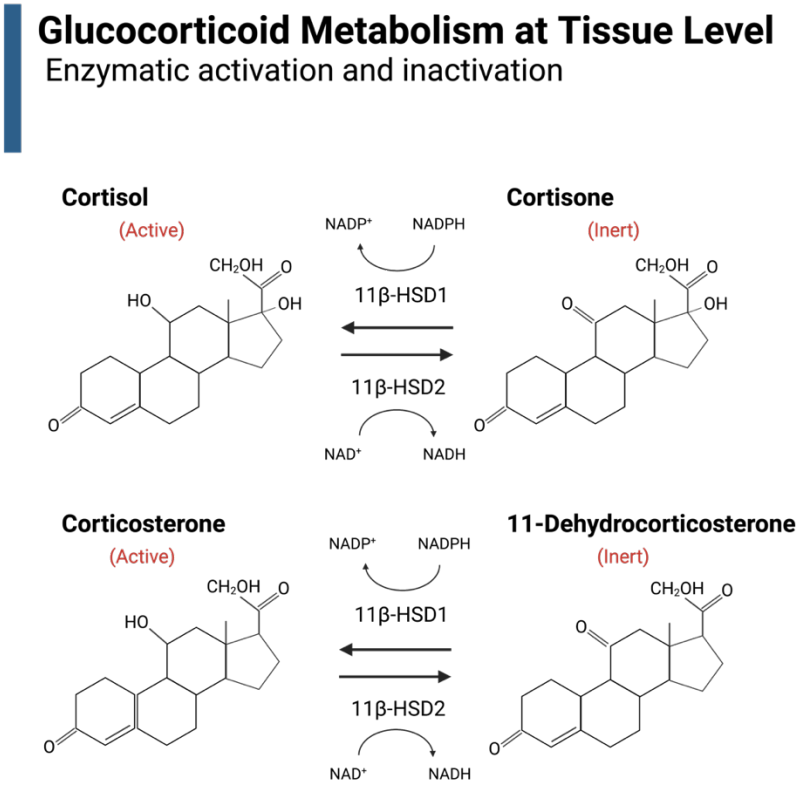

Figure 5. The local availability of GCs in tissues is controlled by the two 11β-HSD enzymes. In most mammals, the interconversion of cortisol and cortisone, occurs by enzymatic oxidation or reduction in the carbon at position 11. In rodents, corticosterone and 11-dehydrocorticosterone have similar structures to cortisol, but lack an α-hydroxyl group in the carbon at position 17. The 11β-HSD1 (encoded by the *HSD11B1* gene) acts predominantly as an NADP(H)-dependent reductase to generate active cortisol, but it is a bi-directional enzyme, acting depending on the cell redox state. In the steroidogenic cells of reproductive tissues, such as the ovary, the oxidative activity of 11β-HSD1 is promoted, favoring the GC activation. On the other hand, 11β-HSD2 (encoded by the *HSD11B2* gene) is an NAD^+-dependent enzyme that unidirectionally inactivate GCs.

GLUCOCORTICOIDS IN REPRODUCTION: A MATTER OF BALANCE?

Mammalian reproduction is a complex and narrowly regulated function that is absolutely under hormonal control. The reproductive hormonal axis, also called hypothalamic-pituitary-gonadal (HPG) axis, is essential to maintain the equilibrium in the reproductive system, which can be altered by different stimulus. The inhibitory effect of stressful situations on the animal reproduction, associated to the so-called fight-or-flight biological response (Wingfield & Romero, 2015), have been taken for granted over the years. However, little attention compared to other steroids has been given to the necessity of balance between high and low GC levels in reproduction, which is able to discriminate fertility and infertility (Whirledge & Cidlowski, 2017). Consequently, elucidating the molecular mechanisms behind this equilibrium should be of interest for research, since the GC regulation in the reproductive organs is complex and insufficiently understood nowadays. As previously mentioned, most of the GC actions are mediated throughout the GC receptor, which acts as a transcription factor that has an important influence by modulating the expression of a considerable number of genes, which in humans encompasses 10-20% of the whole genome (Oakley & Cidlowski, 2011). In the female reproductive tract, GCs can influence the ovarian physiology by the regulating the functions in the ovary, the granulosa cells and the oocytes. Cortisol injection impaired mouse oocyte competence by activating apoptosis of ovarian cells via Fas system (Yuan *et al.*, 2016). However, GC supplementation during the IVM of bovine oocytes increased the oocyte developmental competence and blastocyst developmental rates (da Costa *et al.*, 2016; Santana *et al.*, 2014). According to literature, very different effects are found between species when oocytes and embryos are exposed to GCs during the early preimplantation development *in vitro* (da Costa *et al.*, 2016; Gong *et al.*, 2017; Scarlet *et al.*, 2017; Tetsuka *et al.*, 2016; Yang *et al.*, 1999). Currently, the available

knowledge regarding the role of endogenous GCs in the physiology relies on exposure studies, and may not represent the real situations that occur at physiological levels both in the oviduct or the uterus. Therefore, understanding the GC signaling pathway may be essential for unraveling the mechanisms behind the stress effects on fertility and reproductive success, especially in females.

Furthermore, the unbalance in ROS and mitochondrial damage may affect the great number of signaling pathways involved in energy production, lipid biosynthesis, apoptosis or steroid hormones (Agarwal *et al.*, 2005; Kokkinopoulou & Moutsatsou, 2021). There is an increasing number of recent studies reporting genic, transcriptomic and protein changes in expression related to vitrification and stress *in vitro*, involving pathways related to cell cycle, mitochondria, metabolism, fertilization and many more (Eroglu *et al.*, 2020; Jia *et al.*, 2020; Ma *et al.*, 2018; Wang *et al.*, 2017). Since the precise roles of some of these pathways in reproductive biology are not sufficiently understood, it is currently arduous to address the real repercussion of these findings. From this perspective, there are still a lot of questions to be answer in terms of identifying gene expression patterns of pathways with potential relevance in fertility. Their influence in the specific reproductive steps of each species seems to be one of the key next steps in reproductive research (Schimenti & Handel, 2018). In this sense, the GC regulation is a great example of a pathway insufficiently comprehended in fertility.

GLUCOCORTICOID RECEPTOR

Released GCs can diffuse through the cell membranes, where can bind to the GC receptor, also called GR or NR3C1, which is encoded by an equally named gene (*NR3C1*). This receptor belongs to the nuclear receptor (NR) super-family, which includes another corticosteroid receptor as well, the mineralocorticoid receptor

(NR3C2), but also sex steroid receptors, such as the androgen receptor (AR or NR3C4), the estrogen receptors (ERα/ERβ or NR3A1/NR3A2), and the progesterone receptors (PR or NR3C3). Both NR3C1 and NR3C2 are paralogous hormone-regulated transcription derived by duplication from a common ancestral corticosteroid receptor (CR) around 450 million years ago (Baker *et al.*, 2013; Carroll *et al.*, 2011). According to literature, while NR3C2 binds both mineralocorticoids and GCs, NR3C1 has exclusive specificity for the activation by GCs (Baker *et al.*, 2013). The corticosteroid-derived receptors share a common structure of protein domains, which requires the binding of a steroid ligand, and the binding to DNA in the nucleus for activating gene transcription (Figure 6).

The *NR3C1* gene undergo alternative splicing, resulting in a vast amount of different isoforms that differ only in the C-terminal region, although the majoritarian form in tissues is the GR-α, which is supposed to mediate the most biological actions of GCs (Cruz-Topete & Cidlowski, 2014). The known isoforms (GR-α-A, GR-α-B; GR-α-C1; GR-α-C2; GR-α-C3; GR-α-D1; GR-α-D2; GR-α-D3; GR-β; GR-γ; GR-A; GR-P) bind to GCs with different affinities, and several of them can be co-expressed at the same time (Oakley & Cidlowski, 2013). The receptor is ubiquitously expressed by nucleated cells and present in most of the organs, although the expression patterns seem to be different across the tissues (Lattin *et al.*, 2015). Moreover, the GC receptor could be subjected to posttranslational modifications, including phosphorylation, ubiquitination, acetylation, methylation, nitrosylation, nitration, and SUMOylation, which may influence the transcriptional activity (Scheschowitsch *et al.*, 2017). These features add even more complexity to the GC receptor signaling, influenced by tissue-specific expression patterns, isoforms, posttranslational modifications and glucocorticoid availability.

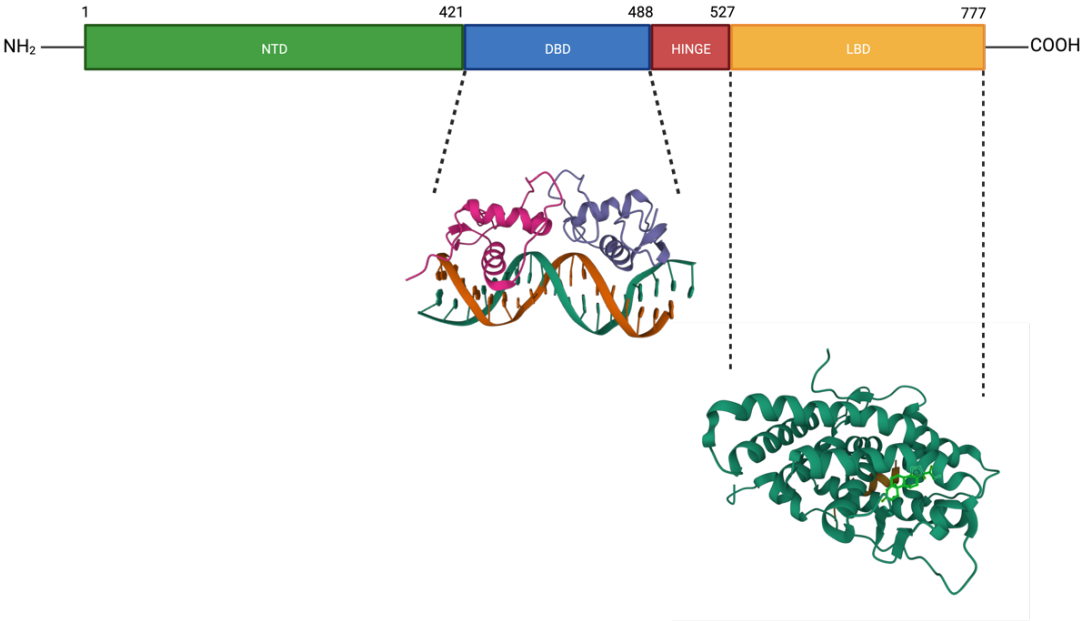

Figure 6. Linear domain structure of the GC receptor NR3C1. Typical CRs are structured on a hinge that gives structural flexibility (HINGE); a DNA-binding domain (DBD) formed by two zinc-fingers, responsible for the interactions with the DNA and cofactors; a ligand-binding domain at the C-terminal region, which interacts with the steroid hormones but also with cofactors and other transcription factors (LBD); and a ligand-independent domain at the N-terminal region (NTD) (Sacta *et al.*, 2016; Vandevyver *et al.*, 2014), important for the activation of the transcriptional activity and the most variable among species and isoforms (Kumar & Thompson, 1999). Here is depicted the length of the predominant and best studied isoform of NR3C1, the alpha, which in humans has 777 amino-acids. The three-dimensional structures of the DBD and LBD of NR3C1, bound to DNA, and cortisol, respectively, are also represented. The structures were obtained from the RCSB Protein Data Bank (PDB) identifiers: 1R4O and 4P6X.

GLUCOCORTICOID RECEPTOR MECHANISM OF ACTION

When it is not ligand bound, the NR3C1 receptor is situated in the cytosol as part of an inactive multiprotein complex, including cofactors, heat shock proteins (HSPs), and immunophilin FK506-binding protein 51. The immunophilin FK506-binding protein 51, also known as FKBP51 or FKBP5, has peptidyl-prolyl *cis*-trans isomerase activity, involved in folding changes and the activities of other co-chaperons. Upon steroid ligand-binding, the conformation of the multiprotein complex changes, and FKBP5 is replaced by the immunophilin FK506-binding protein 52, also called FKBP52 or FKBP4 (Zannas *et al.*, 2016). As a result of these conformational changes, FKBP4 recruits dynein, motor protein that favors the translocation of the receptor to the nucleus, promoting NR3C1 movement towards the nucleus. Once in the nucleus, the complex binds to GC responsive elements (GREs) and interact with other transcription factors, being responsible for stimulating the expression of a vast number of genes, and/or the downregulation of some other genes (Ratajczak *et al.*, 2015). This regulation also includes a negative feedback mechanism in which both the expression of the GC receptor, and also the GC hormone release, are also modulated at local and systemic level, respectively (Denny *et al.*, 2000). In particular, NR3C1-mediated transcriptional activity stimulates the expression of FKBP5, which replaces FKBP4 (Davies *et al.*, 2002). Thus, FKBP5 reduces the NR3C1 sensitivity to ligand binding and blocks the NR3C1-complex interaction with the dynein motor protein, hindering the translocation of the complex to the nucleus, and consequently stopping the transcriptional regulatory activity of NR3C1 (Wochnik *et al.*, 2005). A visual representation of the NR3C1-mediated mechanism of GC actions in the nucleus is shown in Figure 7.

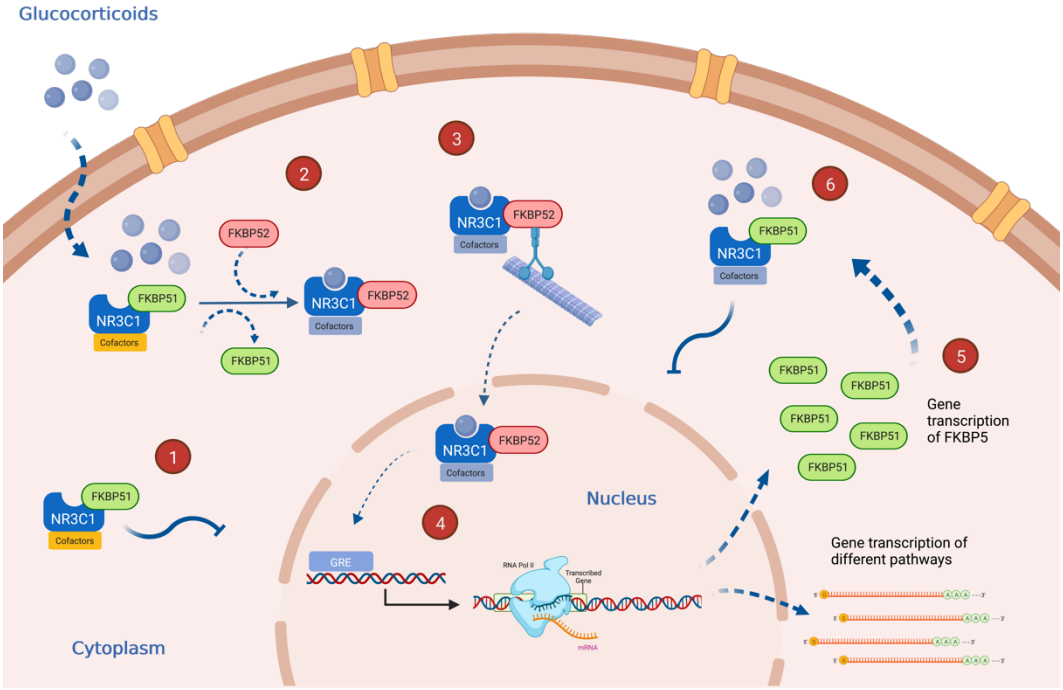

Figure 7. The mechanism of NR3C1 actions in the nucleus. Cytosolic inactive NR3C1 receptor complex bound to FKBP51 (**1**). GC ligand binding induces a conformational change in the NR3C1 complex, FKBP51 is replaced by FKBP52 (**2**). FKBP52 recruits dynein favoring the translocation of the NR3C1 complex to nucleus (**3**). The NR3C1 complex binds to GC responsive elements (GRE), stimulating gene transcription (**4**). NR3C1-mediated transcription stimulates *FKBP5* gene expression, inducing FKBP52 replacement by FKBP51 (**5**). The NR3C1 sensitivity to GCs is reduced by FKBP51, stopping further translocation to the nucleus (**6**).

MAMMALIAN REPRODUCTION

One of the most important definitions of the biological species concept coined in the last century, relies on reproductive isolation between species (De Queiroz, 2005). In this sense, reproduction is a fundamental matter for distinguishing sexually-reproducing species. It is therefore not rare to find reproductive features and traits that are unique for a certain species, or rather diverse within phylogenetically close families. These differences can be present in many forms, including the anatomy of the reproductive organs, the characteristics of the gametes and embryos, the reproductive cycle, the number of ovulations, the mechanism triggering ovulation, and other aspects related to the biology of reproduction (Katkov, 2012). Due to these particularities, potential similitudes between species in some organs or tissues cannot be taken for granted when concerning reproductive physiology, since they would often differ. From this perspective, it seems clear that adapting protocols and ARTs to the physiology of each individual species could be an asset for addressing the current challenges in the field of reproductive biology. Such approaches can be customized for reproductive optimization in domestic species traditionally used for commercial purposes, such as the bovine, the porcine, or the rabbit, but also for biodiversity conservation of threatened and endangered species and breeds. In this context, there is still a long journey for reproductive scientists until achieving optimal ARTs for most species. Thereon, it seems crucial to unravel the biological mechanisms behind the different events taken place during reproduction, often insufficiently understood even for domestic species, normally more accessible for research purposes compared to wild biodiversity.

COMPARATIVE MAMMALIAN FEMALE REPRODUCTION

Most mammalian reproductive events, occur inside of the female reproductive tract, starting from ovulation, reception of the male ejaculate, oocyte(s) fertilization, preimplantation embryonic development, implantation, and finishing with gestation, until parturition is achieved. The origin of the female reproductive tract is the differentiation of the Müller ducts, feature that is shared between eutherian mammals. Therefore, the main structure of the female reproductive tract of this phylogenetical group is common, having paired ovaries, connected to oviducts, uterus, cervix and vagina. However, diverse differences are found between domestic species, which can translate into functional differences of the reproductive events taking place in each of them.

ESTROUS CYCLE

Besides some primates (including humans), three bats (*Carollia perspicillata, Glossophaga soricina, Molossus ater*), one rodent (*Acomys cahirinus*), and one elephant shrew (*Rhynchocyon petersi*), in all described mammalian species, the stages of the reproductive cycle are defined by the estrous cycle (Catalini & Fedder, 2020). The female pig (*Sus scrofa domesticus*) and the cow (*Bos taurus*) are polyestrous species, having several heat periods during the year, which last around 18-24 days. Although periods of increased sexual activity have been described (4-10 days) (Caillol *et al.*, 1983), there is not a regular estrous cycle in the rabbit (*Oryctolagus cuniculus*), being an induced ovulator in which ovulation is only triggered by mating, a similar stimuli, or an exogenous pharmaceutical supplementation (Geyer *et al.*, 2016). The puberty is reached at 5-7 months of age in the female pig, at 4-8 months of age in the does, while this happens at 12-15 months of age in the cow, although other factors such as nutrition, breed, and

the presence of a male, could variate this parameter (Quesenberry & Carpenter, 2012). In the pig, the estrous lasts from 24-72 h, and ovulation is continual, occurring at 30-40 h from the estrous start. Bilateral ovulation typically encompasses the release of 20-30 mature follicles between 8-10 mm in diameter, as the pig is a polyovulatory species. In cattle, the estrous has a length of 8.5-14 h, and the fate of the dominant follicle depends on the phase of the estrous cycle, as ovulation in cattle is mono-ovulatory and spontaneous. Typically, three waves of follicular growth happen during the cycle, however, only the dominant follicle occurring during the follicular phase of the cycle will be ovulated. The rabbit, like the pig, is also a polyovulatory species, however the ovulation is induced. The development of 5-10 follicles per ovary occurs in waves, that produce estrogens for 12-14 days. If there is a triggering stimulus, a neuro-endocrine reflex induces the release of GnRH which may in turn produce an LH peak, causing the ovulation of 5-10 follicles in each ovary, about 10-13 h after the stimulus is produced. In the absence of stimulus, the follicles degenerate and ovulation is not produced. In these species, the empty follicles become corpus luteum, in which the released progesterone blocks new follicular waves (Rosselli *et al.*, 1998).

OVIDUCTAL TRANSPORT

While the oviduct was considered for a long time a mere connection between the ovary and the uterus, its role in important functions has been proven in mammals, including, oocyte transport, fertilization and early embryo development, gamete transport and sperm reservoir (Pérez-Cerezales *et al.*, 2018). The oocytes are released into the infundibulum fimbria and transported throughout the oviduct, with the aid of the luminal epithelium ciliary contraction, helped by the estrogens, and the cumulus cells surrounding the oocyte. The oviduct segment is continued

in the ampulla, which is the fertilization site in most domestic species, typically taking place around 3-14 h after ovulation, depending on the species (Hunter, 1974; Hunter & Nichol, 1988). When the oocyte arrives to the oviduct, it is attached to the oviductal epithelium and the zona pellucida (ZP) becomes exposed to the oviductal fluid, important for fertilization and for the embryo-maternal interaction. Moreover, proteins from the oviductal fluid, such as OVGP1, and heparin-like glycosaminoglycans (GAGs) play a role modifying the ZP before fertilization, helping in the control of polyspermy of porcine and bovine oocytes (Coy *et al*., 2012). After fertilization, the first cleavage of the embryo (or embryos) is achieved, and cell divisions continue during the journey throughout the isthmus. In this region, the embryos are physically retained until the uterus is prepared for further embryo development. The time that the early embryo spends in the oviduct could differ depending on the species, ranging from around 3 days in the rabbit, 3-4 days in the pig, and 4-5 days in cattle, when the embryos have a few cells, or they are reaching the morula stage. The utero-tubal junction has functions regulating the sperm passing through on their way to the oviduct, while also providing a barrier to infections, and acting as a sperm reservoir in some species (Coy *et al*., 2008).

UTERINE TRANSPORT

The uterus is well known for being the region where fetal development occurs. Nevertheless, the uterus has also a role in some more essential functions in the mammalian reproductive events. One of the these functions is the barrier against potential infections stablished in the cervix, protecting the uterine environment from the passage of external pathogens. While in humans and dogs the semen is deposited in the vagina, in the pig it is deposited in the cervix, and the sperm

deposition occurs directly in the uterus in horses. Moreover, uterine contractions and peristaltic movements help sperm and transport throughout the uterine environment towards the oviduct. The anatomy of the female reproductive tract of the cow and the doe is represented in **Figure 8**.

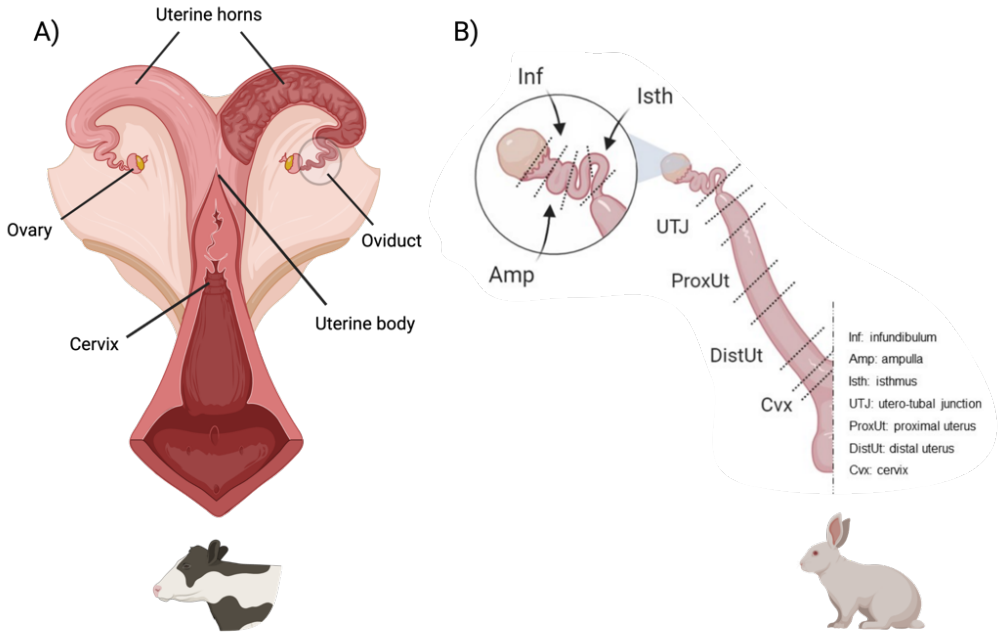

Figure 8. (**A**) Representation of the cow reproductive tract. The most prevalent uterus in mammals has a bicornuate shape, formed by two uterine horns that converge in a simplex uterine body, and a cervix (Fischer *et al.*, 2012). Monotocous species, such as the bovine and the horse, have uterine horns that are less developed compared to the polytocous species, such as the pig, where long horns are followed by an small uterine body and a long cervix, shaped by alternated interdigitated thick pads forming cervical rings. (**B**). Representation of a (half) female rabbit reproductive tract, specifying the different anatomical regions. The amplified area corresponds to the oviductal region. The rabbit female has duplex functionally independent uteruses and cervixes, but a simplex vagina.

A few days after fertilization has occurred, the embryos enter the uterus and continue the early development. Depending on the species, the blastocyst can quickly attach to the uterine wall or, like in the case of bovine, stay in the

uterus for weeks before implantation occurs (Blomberg *et al.*, 2008). Apart from regulating implantation, the uterus is also the region where the placentation takes place, and afterwards, when the fetus has completed the intrauterine development, parturition (Cha *et al.*, 2012).

COMPARATIVE MAMMALIAN MALE REPRODUCTION

For males, having access to mating often requires energetically demanding investments and high risks for individual survival. This biological investment includes a set of behavioral, physiological, and morphological adaptations, such as, among others, far away migrations, incarnate competitive fights with rivals, courtship rituals involving visual, vocal or chemical signals for the female (Andersson, 1994). Across evolution, different biological strategies for reproductive purposes have developed across the animal world in order to achieve fertilization and ensure offspring. In external fertilization, which is evolutionary ancestral, the gametes are subjected to "selection" by being exposed to a great number of potential environmental threats, as they could be damaged, eaten or lost in the flow. In contrast, reproduction in higher animals, such as the mammals, evolved towards internal fertilization, where the gametes are protected from external agents, but come into contact with the selective female environment. This contact produced during the mammalian reproduction, is not only limited to the gametes, but also to the effect of the male penis during copulation, and the influence of the spermatozoa and the seminal plasma contained in the ejaculated. The interaction of all these factors give rise to a sort of complex physiological responses in the female tract which are not fully understood nowadays.

MATING INFLUENCE IN THE FEMALE TRACT

The anatomy of the penis is quite complementary to the female reproductive tract, as the aforementioned case of the pig, showing a spiral-shaped penis, which perfectly matches the sow uterine cervix. Thus, during the act of copulation neuro-electrical signals in the vagina and uterus seem to be induced due to the sensorial stimulation produced by penile buffeting (Alvarez-Rodriguez *et al.*, 2020; Northrop *et al.*, 2010). These afferent signals are transported through the hypogastric, pelvic and pudendal nerves and reach neurons in the limbic system and the hypothalamus (Parada-Bustamante *et al.*, 2016), where this information can be received (Brauer & Smith, 2015). The efferent neural pathways of these signals have been related to a neuroendocrine response that involves the release of oxytocin, progesterone, prolactin and other neuromodulators, inducing changes at protein and gene expression level in the female tract (Álvarez-Rodríguez *et al.*, 2020; Guevara-Guzmán *et al.*, 2001; Shafik *et al.*, 2006) that consequently influence the function of the subsequent reproductive events, including angiogenesis (Krawczynski & Kaczmarek, 2012), sperm tolerance (Álvarez-Rodríguez *et al.*, 2020), sperm storage (Almiñana *et al.*, 2014), oviduct motility (Shafik *et al.*, 2006) and oocyte transport (Orihuela *et al.*, 2001).

At the end of copulation, the male copulatory organ deposits the whole ejaculate inside the female, bathing the different tissues of the internal reproductive tract in the coitus. The location where the ejaculate is released can be different depending on the species. Great differences can also be found in the ejaculate features. The boar deposits 50-60 billion spermatozoa in a volume of seminal plasma that can be about 250-500 mL, due to the large accessory glands secretions (Constantinescu & Schatten, 2017; Rath *et al.*, 2016). The boar ejaculate is released in fractions, with most of the epididymal proteins present in the first

portion of what has been called the sperm-rich fraction, and the seminal plasma proteins prevailing in the rest of the sperm-rich fraction ejaculate volume (Rodríguez-Martínez *et al.*, 2009). In contrast, the bull and the rabbit ejaculates may present a volume that is about 1.5-12 mL (Schenk, 2018; Wiebke *et al.*, 2021) and 0.5-2 mL (Bezerra *et al.*, 2019; Brun *et al.*, 2016), respectively, containing around 800 and 200-600 millions of sperm cells. However, great differences in those numbers can be found in the literature, as a big number of factors such as season, nutrition, age or breed can considerably modify this parameter.

SPERM TRANSPORT THROUGHOUT THE FEMALE TRACT

Male competition seems to be very related to reproduction, as it is the female that normally chooses the partner for copulation, and fights between males for mating are common in different species. Nevertheless, reproductive selection and competition continues also at cellular level (Lüpold *et al.*, 2020). The spermatozoa are highly selected during their journey across the uterus towards the fertilization site, and even cryptic female choice of sperm has been described (Fitzpatrick *et al.*, 2020). The spermatozoa contained in the semen fluid start a journey in the vagina (or the uterus) all across the female reproductive tract towards the oocyte. During the sperm travel, the spermatozoa must pass selection steps inside the female reproductive tract, where at the same time, certain tolerance is promoted in order to allow the sperm cells to achieve fertilization. In species where the semen is deposited in the vagina, the cervical mucus is a great barrier for spermatozoa with low motility, that get trapped in the mucus and are eliminated by neutrophile infiltration (Miller, 2018). In contrast, most of the normal motile sperm cells are able to cross throughout the cervical canal and reach the uterus. In these species, the interactions between sperm and the uterus remain limited,

as no increase in polymorphonuclear leukocytes was found in uterine epithelial cells in contact with ejaculate (Wendt, 2007). However, in the pig the transport of the sperm cells towards the oviduct is helped by contractions of the uterine smooth muscle that seems to be influenced by endocrine regulation. Spermatozoa are transported through the uterine horns and get attached to uterine epithelial cells and granulocytes, producing the activation of pro- and anti-inflammatory cytokines that mediate sperm selection by modulating sperm loss and tolerance.

A profound selection is produced in the utero-tubal junction (UTJ), where the sperm cells are massively retained, and only a lower number of a majority of normal spermatozoa are allowed to enter the lower part of the oviduct, the isthmus (Baker & Degen, 1972). There is storage of spermatozoa in the female tract in what has been called the sperm reservoir (Rodríguez-Martínez *et al.*, 2005), where the lifespan of these cells can be prolonged for no more than two days in most mammals (Holt & Fazeli, 2016). The precise anatomical location may differ between species. While the UTJ seems to act as sperm reservoir in the pig (Rodriguez-Martinez *et al.*, 2001), in most species, the isthmus is the region acting as reservoir for spermatozoa, including cattle (Lefebvre *et al.*, 1995) and rabbit (Overstreet *et al.*, 1978). Sperm cells accessing to the oviduct may be only a few thousands, which is a very reduced number compared to the numbers found in the uterus. However, the cells reaching the oviduct are mainly live, motile, morphologically normal, uncapacitated and acrosome intact spermatozoa (Miller, 2018; Rath *et al.*, 2016). These sperm cells bind to oviductal epithelial cells in the isthmus and the ampulla, mediated by a variety of different receptors, including annexins, such as ANXA1, or HSPs, including HSPA5 or HSPA8 as examples of proteins identified in the cells of both cattle and pig (Mahé *et al.*, 2021), while in rabbit, interactions in this matter remain unknown nowadays. The binding to the

oviductal epithelial cells seems to be a selective process, as capacitation and hyperactivation of motility has been found in the spermatozoa released from this attachment.

In vitro, exposition of spermatozoa to oviductal fluid flushing, or oviductal extracellular vesicles seem to produce an effect in sperm capacitation and activation of motility, at least in in cat, cattle and pig (Alcântara-Neto *et al.*, 2020; Coy *et al.*, 2010; Ferraz *et al.*, 2019; Lamy *et al.*, 2018). The spermatozoa movement is aided by oviductal contractions, but sperm cells also actively swim against the flow of the medium (Hyakutake *et al.*, 2015), guided by rheotaxis (Miki & Clapham, 2013), chemotaxis (Eisenbach, 1999) and thermotaxis (Pérez-Cerezales *et al.*, 2015; Rodríguez-Gil, 2019) towards the fertilization site. Thus, hyperactivated and capacitated spermatozoa reach the cumulus oocyte complex (COC) in the ampulla, swimming through the viscous *corona radiata* of cumulus cells. During the preliminary contact with the zona pellucida, the acrosome reaction is triggered in the sperm, allowing the oocyte penetration and fusing with the oolemma. At this point, the genetic information is incorporated into the oocyte and fertilization is finally achieved. Oviductal selection of sperm also seems to have a direct function in fertilization. The elimination of sperm may be a key feature for the penetrating ability and also promoting monospermy, as the removal of the isthmus was translated in a rise of polyspermy in pig and rabbit (Hunter & Léglise, 1971a; Hunter & Léglise, 1971b).

SEMINAL PLASMA INFLUENCE IN THE FEMALE TRACT

The semen has been assumed for a long time to have the only role of transporting the spermatozoa toward the ampulla to accomplish the primary biological reproductive function, achieve fertilization. However, the ejaculate is a complex

fluid, comprising spermatozoa, but also seminal plasma (Schjenken & Robertson, 2020). Seminal plasma contains a variety of signaling factors, including cytokines, prostaglandins, glycans, nucleic acids, proteins and steroid hormones that are able to elicit a molecular response in the female reproductive tract (Robertson, 2007). This response seems to be a mechanism that is highly conserved through evolution, as comparable effects have been identified across different taxa, including birds, insects, and also, mammals (McGraw *et al.*, 2015). Thus, seminal plasma influence may have a substantial biological value that goes far beyond sperm transport (Druart & de Graaf, 2018). The changes induced are pleiotropic, and include the modulation of the immune response, encompassing an activation of an inflammatory-like response. This response is followed by an anti-inflammatory cascade for the promotion of tolerance towards the sperm cells transport in the female reproductive tract, and also the allowance of the embryo implantation (Álvarez-Rodríguez *et al.*, 2020). As aforementioned, the ejaculate is deposited either in the vagina or the uterus. However, seminal plasma influence is not limited to local effects on the tissues directly exposed to the fluid, but also distant organs, such as the oviduct, the ovary or even into the peritoneal cavity through the abdominal foramen of the infundibulum (Robertson, 2005). Small molecules in the seminal plasma can be released into the uterine vein and transferred to the ovarian artery. These molecules can include ovulation-inducing factors, such as the nerve growth factor β (β-NGF), which can access the HPA axis, triggering ovulation in some species, including the rabbits or the camels (Silva *et al.*, 2011). They can even alter the ovulation dynamics in non-induced ovulatory species (Tanco *et al.*, 2012), as in the pig, where seminal plasma can influence ovulation and corpus luteum formation (O'Leary *et al.*, 2004, 2006).

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HYPOTHESIS

Glucocorticoids (GCs) are steroid hormones that can inhibit reproduction during stress responses, but are also essential at baseline levels for some reproductive events of the female reproductive physiology. Although there are evidences of their importance during ovulation, oocyte maturation or implantation, the role of GCs in reproduction is complex and still unclear. The biological mechanisms involved seem to be importantly regulated, involving the GC receptor NR3C1, and a great number of enzymes and co-factors. Moreover, the GC exposure seems to be considerably different between tissues and species. Thus, a better comprehension of the regulatory pathways of the GC regulation, may be potentially helpful for developing more efficient assisted reproductive techniques and understanding the molecular mechanisms behind the stress impairment of reproduction. In this sense, the study of the regulatory GC expression in bovine oocytes and cumulus during *in vitro* maturation and vitrification, and the supplementation with coenzyme Q10, a endogenous molecule with important antioxidant properties, could improve the knowledge on oocyte management and conservation *ex vivo* for reproductive purposes. In addition, the determination of the changes in the GC regulatory expression across the different tissues of the female reproductive tract of bovine, porcine and rabbit species during reproductive events, such as the estrous cycle, the male-female interactions (natural or assisted), or the early steps of the development, may be essential for understanding of the implications of the GC roles in the animal reproductive physiology.

OBJECTIVES

The general objective of this thesis is to study the role of the gene and protein expression of the glucocorticoid receptor –and other molecules involved in glucocorticoid regulation– in the reproductive function, by determining its modulation in the female reproductive tract during reproductive events *in vivo* and *in vitro*.

The specific objectives are as follows:

I. To evaluate the effects of coenzyme Q10 supplementation during oocyte *in vitro* maturation as a potential molecule for attenuating the negative effects of vitrification.

II. To examine the changes in glucocorticoid and apoptosis expression in bovine oocytes and cumulus cells after *in vitro* maturation, coenzyme Q10 supplementation and vitrification.

III. To determine the changes in glucocorticoid expression in the bovine endometrium and ampulla during different stages of the estrous cycle.

IV. To assess the influence of natural mating, artificial insemination and seminal plasma infusion in the glucocorticoid mRNA expression in the reproductive tract of preovulatory sows.

V. To evaluate the modulation over time of the glucocorticoid receptor mRNA expression in the rabbit female reproductive tract during early reproductive events after mating.

CHAPTER I

Coenzyme Q10 Supplementation during *in vitro* Maturation of Bovine Oocytes (*Bos taurus*) Helps to Preserve Oocyte Integrity after Vitrification

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ABSTRACT

Oocyte vitrification causes less cell stress than slow cooling, but cytoskeletal and spindle alterations may occur, affecting the oocyte competence. *In vitro* maturation (IVM) supplementation with antioxidant molecules has been an strategy used to attenuate this harmful stress. Coenzyme Q10 (Q10) supplementation has previously shown positive effects in bovine and mouse *in vitro* embryo development. The aim of this study was to evaluate the effects of Q10 during bovine oocyte IVM and vitrification. Cumulus–oocyte complexes (COCs) (n = 311) were cultured under standard maturation conditions with 0 μM (control), 25 μM and 50 μM Q10 supplementation. After 22 h, a cohort of 170 oocytes both from the control and from Q10-supplemented groups were vitrified, warmed and returned to incubation until 24 h of maturation, while the rest of the oocytes (n = 141) remained fresh (non-vitrified). Then, oocyte survival was assessed morphologically by stereomicroscopy. Oocytes from all groups were then fixed and stained for assessing cortical granules migration and nuclear stage. High rates of oocyte metaphase II progression and appropriate cortical granules migration as a continuous layer beneath the plasma membrane were obtained both in control and in Q10 groups in non-vitrified oocytes. Results showed that although vitrification has great impact in the survival of IVM bovine oocytes, 50 μM Q10 supplementation significantly improved oocyte survival (p = 0.045) and reduced the premature cortical granules exocytosis, helping to preserve the cortical granules migration pattern (31.3% control vs. 54.5% in 50 μM Q10; p = 0.039), attenuating the negative effects of vitrification.

Keywords: CoQ10; cortical granules; *in vitro* maturation; oocyte; vitrification.

INTRODUCTION

Although vitrification causes less stress to the cells than slow cooling, trauma to the oocyte gamete is not avoided. Some previous studies have demonstrated that supplementing culture media with antioxidant molecules may be a good approach for improving the developmental competence of the oocyte and reduce the stress caused by low temperatures (Hwang & Hochi, 2014). In this sense, coenzyme Q10 (Q10) has been proposed recently as a promising candidate to enhance fertility and embryo developmental rates (Gendelman & Roth, 2012). The capacity of this molecule to maintain continuous cycles of oxidation and reduction makes of it a very effective membrane antioxidant. Thus, supplementation strategies with Q10 have been previously used to improve different parameters of developmental competence in oocytes during *in vitro* maturation (IVM) with successful results in bovine (Gendelman & Roth, 2012). The objective of this study was to examine the effects of Q10 supplementation of bovine oocytes during IVM after vitrification.

MATERIALS AND METHODS

COLLECTION OF OVARIES AND COCs RETRIEVAL

Briefly, the bovine ovaries were collected at a local abattoir, transported to the laboratory, washed with sterile saline solution (38.5°C) and kept at that temperature until finishing the follicular puncture. The oocyte recovery was carried out from 2- to 8-mm follicles, and follicular contents were examined under a stereomicroscope for oocyte collection. The oocytes were washed three times with PBS supplemented with 0.05% (w/v) BSA, glucose (5.55 mM), sodium

pyruvate (0.33 mM; DPBS Glc-Pyr; Gibco) and 1% (v/v) antibiotic and antimycotic solutions (Ab-Am; 10,000 U penicillin, 10 mg streptomycin and 25 mg amphotericin B per mL). COCs were recovered and washed twice in working medium (Medium 199 with Earle's salts, 1% [v/v] HEPES). Only oocytes with more than three CC layers and homogeneity of the ooplasm were selected for this experiment.

IN VITRO MATURATION

After being washed in maturation media (MM; TCM199 supplemented with 20 mg/ml epidermal growth factor, 0.2 mM sodium pyruvate and 1% [v/v] Ab-Am solution), COCs were randomly placed in groups of 20–25 in a 4-well culture dish filled with 500 µl of MM supplemented with Q10 at a final concentration of 0 µM (control), 25 µM or 50 µM. After 22 h, a cohort of the oocytes (control, n = 57; 25 µM Q10, n = 56; 50 µM Q10, n = 57) were vitrified (n = 170), warmed and returned to incubation until 24 h of maturation, while the rest of the oocytes remained fresh (n = 141). After IVM, COCs were recovered and washed twice in DPBS Glc-Pyr. Denudation of the COCs was performed mechanically by gently pipetting.

VITRIFICATION AND WARMING

Vitrification was performed at 22 h of IVM using a commercial carrier device (Cryoloop, Hampton Research, USA), and using vitrification and warming solutions previously described (Kuwayama *et al.*, 2005). Oocytes were then recovered from MM, washed in PBS Glc-Pyr and transferred for 5 min to an equilibrium solution composed by 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulphoxide (DMSO) in PBS supplemented with 20% fetal calf serum (OCM). After that, they were carefully placed in the vitrification solution (VS) composed by 15% (v/v) EG,

15% (v/v) DMSO and 0.5M sucrose in OCM for 30 s. Then, oocytes were rapidly transferred onto the tip of the carrier device with the minimal volume and instantly plunged into liquid nitrogen (LN_2). Warming was carried out by directly immersing the cryoloop into the warming solution (0.5M sucrose in OCM) for 5 min, and then, oocytes were transferred and kept for 5 min in each of the three sequential hydration solutions: 0.25M sucrose, 0.1M sucrose and PBS solutions. After that, oocytes were washed once in MM and returned to the incubation conditions for finishing 24 h of IVM, and then, oocyte survival rate was evaluated morphologically by stereomicroscopy. Non-viable category was assigned if oocytes presented empty or discontinuous zona pellucida, darkened or non-homogeneous cytoplasm, evidences of shrinkage, cytoplasmic vacuolization or non-rounded shape, following previously described criteria (Succu *et al*., 2007). Fresh oocytes were equally assessed after 24 h of IVM.

CORTICAL GRANULES AND NUCLEAR STAINING EVALUATION

Denuded oocytes were immersed in PBS containing 0.4% (w/v) Pronase E for approximately 3 min until the zona pellucida was digested and washed five times with PBS. The oocytes were then fixed in a paraformaldehyde solution at 4% (w/v) in PBS for 30 min at 38.5°C. Afterwards, the oocytes were permeabilized and then stained for cortical granules using fluorescein isothiocyanate-labelled agglutinin (FITC–LCA) and 4′,6-diamidino-2-phenylindole (DAPI) for DNA staining as previously described (Andreu-Vázquez *et al*., 2010). Evaluation of cortical granules migration and nuclear stage was performed for each oocyte. DNA and different sections of every oocyte were photographed in UV epifluorescence microscope and laser confocal microscope. Metaphase II (MII) was evaluated for each oocyte. The MII morphology status was checked according to a previously described

classification (Tseng *et al.*, 2004). Migration of cortical granules was assessed, and their distribution was classified into four patterns according an established classification (Hosoe & Shioya, 1997) that has been previously modified (Andreu-Vázquez *et al.*, 2010).

STATISTICAL ANALYSIS

All results were analyzed by using contingency tables and Fisher's exact test. GraphPad InStat software (3.1a, GraphPad software, San Diego, USA) was used to perform the analysis. This software computes the two-sided *p*-value using the method of summing small *p*-values. In all cases, differences were considered significant at $p < 0.05$.

RESULTS

The vitrification process caused a decline in the morphological oocyte survival for all groups compared to fresh oocytes (control, $p = 0.0001$; 25 µM Q10, $p = 0.0003$; 50 µM Q10, $p = 0.03$). However, oocyte survival of vitrified oocytes from the 50 µM Q10 supplementation group was significantly improved compared to the control one (57.9% vs 77.2%; $p = 0.045$). High rates of oocyte MII progression were found in all groups of fresh oocytes (control: 72.7% ± 4.5; 25 µM Q10: 76.2% ± 3.2; 50 µM Q10: 73.2% ± 1.8), which also had appropriate cortical granules migration (control: 65.9% ± 1.4; 25 µM Q10: 64.3% ± 5.4; 50 µM Q10: 68.3% ± 1.5).

In all groups, vitrification impaired the maintenance of the MII conformation compared to fresh oocytes in all groups ($p < 0.01$; control: 37.5% ±

1.0; 25 µM Q10: 35.3% ± 7.5; 50 µM Q10: 38.6% ± 1.3) although there were no differences between vitrified groups. Also, vitrification damaged the preservation of the appropriate cortical granules migration in control (31.3%; $p = 0.0027$) and 25 µM Q10 (29.4%; $p = 0.003$). However, IVM in presence of 50 µM Q10 helped to maintain the appropriate cortical granules migration pattern as a continuous layer beneath the plasma membrane (54.5%) compared to control (31.3%; $p = 0.039$) and also 25 µM Q10 groups (29.4%; $p = 0.038$), as shown in **Figure 1**.

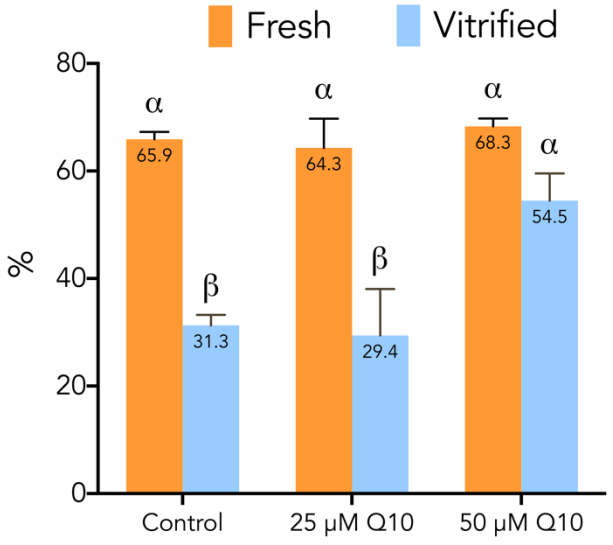

Figure 1. Maintenance of cortical granules as a continuous layer beneath the plasma membrane after oocyte vitrification in presence of 0 µM (Control), 25 µM and 50 µM CoQ10 (control vs 50 µM Q10, $p = 0.039$; 25 µM Q10 vs 50 µM Q10, $p = 0.038$). α-β: Values with different letters are significantly different.

DISCUSSION AND CONCLUSIONS

According to our results, morphological survival rates were significantly impaired due to the harmful effects of vitrification. It has been already described that

vitrification lowered the survival rates of oocytes, which can be explained by thermal, mechanical and chemical factors causing damage of membranes, changes in cortical granules distribution and altered meiotic spindle (Kuwayama *et al.*, 2005). In regard of these results, it seems that Q10 helps in the resistance of the oocyte to deal with vitrification stress, as shown in the 50 μM treatment, where these differences seem to be attenuated when compared to control.

It has been previously stated that vitrification significantly damages cortical granules (Fuku *et al.*, 1995). Interestingly, our results showed that the maintenance of the appropriate cortical granules migration in vitrified and warmed oocytes was significantly improved when the oocytes were matured in the presence of 50 μM Q10, compared to control, and a to lower concentration of Q10 (25 μM), helping to preserve the oocyte integrity after vitrification and reducing the premature cortical granules exocytosis. Inappropriate cortical granules migration caused by stress has been linked with the phenomenon of oocyte ageing, and Q10 may be attenuating this deleterious effect acting as an antioxidant due to its role in the mitochondrial function. In conclusion, oocyte supplementation with 50 μM Q10 during IVM helps to preserve oocyte integrity after vitrification in terms of avoiding undesirable premature cortical granule exocytosis.

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CHAPTER II

Apoptosis and Glucocorticoid-Related Genes mRNA Expression is Modulated by Coenzyme Q10 Supplementation during *in vitro* Maturation and Vitrification of Bovine Oocytes and Cumulus Cells

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ABSTRACT

Oocyte *in vitro* maturation (IVM) and vitrification procedures lead to detrimental effects on the overall oocyte quality. The addition of antioxidants during IVM, such as the coenzyme Q10 (Q10), has been demonstrated to positively impact on the cumulus-oocyte complexes, due to its role in protection from oxidative damage and modulating gene transcription. Furthermore, glucocorticoids (GCs) regulate gene transcription, energy metabolism and apoptosis during the early steps of reproduction. In this sense, most GC actions are mediated by the glucocorticoid receptor (NR3C1), a transcription factor. However, the specific roles of GC in ovarian physiology and oocyte maturation are still unknown. In this regard, a better knowledge on the expression of GC-related and apoptosis-related genes during IVM and cryopreservation procedures could potentially benefit the refinement of assisted reproductive techniques in the bovine species. The present study aims to explore the expression of *NR3C1* mRNA in fresh and vitrified bovine oocytes and cumulus cells in response to Q10 (50 μM), and the effect of cortisol addition (0.25 μM, 0.50 μM) on the expression of NR3C1. We also studied the mRNA expression of *NR3C1*-related genes belonging to the GC regulation pathway, such as hydroxysteroid dehydrogenases (*HSD11B1*; *HSD11B2*), immunophilins (*FKBP4*; *FKBP5*), signal transducers and activators of transcription (*STAT3*; *STAT5A)*, the mineralocorticoid receptor (*NR3C2*), and to the apoptosis pathway, such as the anti- (*BCL2*) and pro-apoptotic (*BAX*) mRNA transcripts in oocytes and cumulus cells 1) after IVM, and 2) after vitrification, both in presence or absence of Q10 supplementation during IVM. Our results show that there is an increase in the NR3C1 receptor expression after vitrification of oocytes, but not after exogenous cortisol supplementation during IVM. In addition, Q10 reduces the mRNA expression of *HSD11B1* and *FKBP5* in oocytes at levels of immature

oocytes (*HSD11B1* mRNA expression also in cumulus cells), and the *BAX*:*BCL2* ratio mRNA expression. After vitrification in the presence of Q10, *HSD11B2* mRNA expression increases in cumulus cells, while *HSD11B1*, *FKBP5* and *BAX*:*BCL2* mRNA expression decreases significantly both in oocytes and cumulus cells. In conclusion, our results show for the first time the effect of IVM, vitrification and Q10 supplementation on the mRNA relative expression of GC-related and apoptosis genes, and the effect of vitrification in the protein expression of NR3C1.

Keywords: *NR3C1*; *HSD11B2*; *FKBP5*; qPCR; cattle; antioxidant.

INTRODUCTION

Ex vivo management of oocytes involves their exposure to artificial *in vitro* maturation (IVM) conditions and, frequently, to procedures conceived to help reproductive purposes, such as oocyte cryopreservation (Vajta & Kuwayama, 2006). Although IVM conditions are designed to mimic the female environment *in vivo*, and cryopreservation techniques are rapidly improving over time, they still are stressful events for the oocytes (Combelles *et al.*, 2009). In the last years, vitrification has significantly improved the oocyte cryopreservation outcomes for assisted reproduction purposes, such as human fertility, *ex situ* germplasm conservation, and animal breeding programs (Cobo *et al.*, 2016; Tharasanit & Thuwanut, 2021). Despite being more efficient and reliable than slow freezing (Chian *et al.*, 2004), vitrification induces sublethal damage, including cytoskeletal and spindle alterations (García-Martínez *et al.*, 2020). In this sense, the yields of blastocysts transferred after IVM, *in vitro* fertilization, and *in vitro* culture of vitrified bovine oocytes still remain suboptimal (Mogas, 2019). Lower oocyte survival rates after vitrification can be explained by thermal, mechanical, and chemical factors causing damage to membranes, anomalies in cortical granules distribution, and altered meiotic spindle (Mogas, 2019). Moreover, vitrification has a negative impact on the mitochondrial function of the oocyte (Gupta *et al.*, 2010), causing damage to the mitochondrial membrane, and low electron density of the mitochondrial matrix, which alters the intracellular anti-oxidative system, leading to ATP depletion, oxidative stress and reactive oxygen species (ROS) increase (Dai *et al.*, 2015; Zhao *et al.*, 2011). The oxidative imbalance and ROS increase can induce apoptosis in the female gamete, impairing the oocyte competence and compromising assisted reproduction outcomes (Combelles *et al.*, 2009; Ott *et al.*, 2007).

Different strategies have been designed to overcome the detrimental effects caused by oxidative stress, including antioxidant supplementation during IVM (Iwata, 2021; Sprícigo *et al.*, 2017). Coenzyme Q10 (Q10) is a lipophilic benzoquinone located in the electron transport chain of the inner mitochondrial membrane (Gendelman & Roth, 2012), that plays a pivotal role in the cell as an endogenous antioxidant, controlling cellular redox and energy metabolism (Crane, 2001). Q10 is a pleiomorphic molecule that acts by altering diverse signaling pathways and influencing gene transcription (Quinzii *et al.*, 2010). Q10 supplementation during IVM improves oocyte developmental competence (Stojkovic *et al.*, 1999), reduces post-meiotic aneuploidies (Ma *et al.*, 2020), and restores mitochondrial function (Ben-Meir *et al.*, 2015). Moreover, previous studies from our group confirmed a significant improvement in oocyte survival after vitrification in the presence of Q10, reducing the premature cortical granules exocytosis, and helping to preserve an adequate cortical granules migration pattern (Ruiz-Conca *et al.*, 2017).

On the other hand, glucocorticoids (GCs) are essential mediators in cell signaling (Geraghty & Kaufer, 2015). The GC receptor, NR3C1, can bind to DNA in the nucleus, modulating pathways related to transcriptional activity, apoptosis modulation, mitochondrial activity, lipid and carbohydrate metabolism, and stress response (da Costa *et al.*, 2016; Datson *et al.*, 2008). The regulation of GC-related genes has been revealed as relevant for the reproductive function (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020; Ruiz-Conca, Gardela, Martínez, *et al.*, 2020; Whirledge & Cidlowski, 2017). The NR3C1 was strongly expressed in oocytes of primordial and antral follicles (Pontes *et al.*, 2019). In addition, *NR3C1* knockdown inhibited the expansion of the cumulus granulosa cells, which may indicate an important role for the receptor in the regulation of follicular processes via paracrine signaling (Ravisankar *et al.*, 2021). Moreover, oocyte sensitivity to GCs

supplementation during IVM remains controversial due to the reported differences regarding their effects across domestic species (Gong *et al.*, 2017). Those differences could be linked to the action of NR3C1, but also to the presence of enzymes involved in the GC metabolism, such as HSD11B1, catalyzing the conversion of cortisone to active cortisol, or HSD11B2, in charge of oxidation of cortisol into inactive cortisone (Michael *et al.*, 2003). In addition, NR3C1 requires cofactors for modulating gene transcription in the nucleus (Wang & Harris, 2015), such as the immunophilins FKBP4 and FKBP5, involved in regulating the intracellular traffic by interacting with the NR3C1 mature hetero-complexes (Kang *et al.*, 2008).

The present study aims to map the changes in the relative expression of the NR3C1 receptor and related genes in response to exogenous challenges, such as cortisol presence, IVM, and vitrification, and whether those changes could be modulated by Q10 supplementation. For this purpose, we analyzed the mRNA expression of GC-related (*NR3C1*, *HSD11B1*, *HSD11B2*, *FKBP4*, *FKBP5*, *NR3C2*, *STAT3*, and *STAT5A*) and apoptosis-related (*BCL2*, *BAX* and *BAX*:*BCL2* ratio) genes on fresh and vitrified bovine oocytes and cumulus cells.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and reagents included in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

BOVINE COCs COLLECTION AND IVM

Bovine ovaries from post-pubertal heifers (12-18 months) were obtained from a local slaughterhouse (*Escorxador de Sabadell,* Sabadell, Barcelona, Spain) and transported to the laboratory at 35-37°C in 0.9% saline solution (NaCl) in less than 1 h. Only reproductive organs from heifers in follicular phase were used for the collection of the ovaries. The contents of small antral follicles (6-8 mm) were aspirated by means of a low-pressure vacuum pump attached to an 18-G needle. The cumulus-oocyte complexes (COCs) included in the study showed more than three compact layers of cumulus cells and a homogeneous cytoplasm. Fifty COCs per well were randomly distributed in 4-well culture dishes (Nalge Nunc International, Rochester, NY, USA) and cultured following the conditions established in each experimental design, based on IVM in 500 µL for 24 h at 38.5°C in 5% CO_2 humified air atmosphere. The IVM medium was composed of TCM-199 with Earle's salts, L-glutamine, and sodium bicarbonate supplemented with 10% (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 µg/mL gentamicin.

EXPERIMENTAL DESIGN

EXPERIMENT 1: EFFECT OF CORTISOL DURING IVM ON NR3C1 PROTEIN EXPRESSION AFTER VITRIFICATION

Groups of 50 COCs were randomly distributed in six experimental groups and *in vitro* matured in: (1) Control: IVM medium; (2) Cortisol 0.25 µM: IVM supplemented with 0.25 µM of exogenous cortisol; (3) Cortisol 0.50 µM: IVM supplemented with 0.50 µM of exogenous cortisol; (4) Control Vitri: IVM and vitrification/warming; (5) Cortisol 0.25 µM Vitri: IVM supplemented with cortisol

(0.25 µM) and vitrification/warming; (6) Cortisol 0.50 µM Vitri: IVM supplemented with cortisol (0.50 µM) and vitrification/warming. After IVM, oocytes in groups of 50 were snap frozen in liquid nitrogen, and stored at -80°C until protein extraction and Western blot analyses for relative protein expression of NR3C1 were conducted. Three biological replicates were performed for this experiment.

EXPERIMENT 2: EFFECT OF Q10 SUPPLEMENTATION ON IVM

Groups of 50 COCs were randomly distributed in three experimental groups: (1) Pre-IVM: immature oocytes at germinal vesicle stage; (2) IVM: IVM medium; (3) Q10 IVM: IVM medium supplemented with Q10 (50 µM). COCs from all treatment groups were denuded by gently pipetting (PIPETMAN ® P100L, Gilson, Spain) to separate oocytes from cumulus cells. After, oocytes in groups of 50 and, separately, their cumulus cells, were snap frozen in liquid nitrogen, and stored at -80°C until RNA isolation and qPCR analyses for relative mRNA expression were conducted. Three biological replicates were performed for this experiment.

EXPERIMENT 3: EFFECT OF Q10 SUPPLEMENTATION ON VITRIFICATION

Groups of 50 COCs were randomly located in two experimental groups: (1) Control IVM: *in vitro* matured COCs; (2) Q10 IVM: COCs *in vitro* matured in IVM medium supplemented with 50 µM exogenous Q10. After 20 h of IVM, half of the COCs on each experimental group were vitrified/warmed. After warming, COCs were allowed to recover for 4 h in the corresponding maturation medium. After finishing the 24 h of IVM, COCs were denuded by gently pipetting to separate oocytes from cumulus cells. After, oocytes in groups of 25 and, separately, their cumulus cells, were snap frozen in liquid nitrogen, and stored at -80°C until RNA

isolation and qPCR analyses for relative mRNA expression were conducted. Three biological replicates were performed for this experiment.

DENUDATION OF COCs, VITRIFICATION AND WARMING

After 20 h of IVM, COCs were partially denuded by gentle pipetting and vitrified following a previously described protocol (Kuwayama *et al.*, 2005) with minor modifications. First, COCs were transferred for 9 min into an equilibrium solution (TCM199-HEPES supplemented with 20% (*v/v*) FBS, 7.5% (*v/v*) ethylene glycol (EG) and 7.5% (*v/v*) dimethyl sulfoxide (DMSO)) at room temperature. After equilibration, COCs were transferred for 30 s into a vitrification solution (TCM199-HEPES supplemented with 15% (*v/v*) EG, 15% (*v/v*) DMSO, and 1 M sucrose). Vitrification was performed in groups of 5 COCs in a small drop of vitrification solution placed in a commercial cryopreservation carrier device (Cryoloop, Hampton Research, USA), which was immediately plunged in liquid nitrogen. For warming, the device containing the COCs was directly immersed into a TCM199-HEPES medium supplemented with 1 M sucrose, at 37°C. After 5 min, COCs were transferred to a TCM199-HEPES medium supplemented with decreasing concentrations of sucrose (0.5 M, 0.25 M, and 0 M) for 5 min in each of them. After warming, COCs were transferred back to the IVM plates at 38.5°C in 5% CO_2 humified air atmosphere, allowing them to recover for 4 additional hours.

Afterward, COCs were then washed in a phosphate buffer saline solution (PBS), and the oocytes were completely denudated from cumulus cells by gentle pipetting. Oocytes were harvested and the remaining medium from each experimental group was centrifuged in separated cryotubes to obtain the cumulus cells (500× g, 3 min, room temperature) after removing the supernatant. Groups of 25 oocytes each were transferred into cryotubes with a minimum volume of

medium. Both cryotubes containing oocytes and cryotubes containing cumulus cell pellets were immediately immersed in liquid nitrogen and kept at -80°C until further analysis.

PROTEIN EXTRACTION AND WESTERN BLOTTING

For protein extraction, the denuded oocytes were homogenized by periodically using vortex and pellet pestle in commercial lysis buffer (RIPA, Thermo Scientific™, Fisher Scientific, Madrid, Spain) at 4°C during 1 h and centrifuged for 10 min at 4°C (13000× g). The protein concentration was obtained by the DC™ Protein Assay Kit (Bio-Rad Laboratories, Kabelsketal, Germany), using bovine serum albumin as a standard reference. For each sample, a protein quantity of a maximum of 10 µg was mixed with 4X sample buffer and denatured for 10 min at 70°C. Protein extracts were then loaded into 4%–20% SDS-PAGE gels (Bio-Rad Laboratories, Kabelsketal, Germany), for electrophoresis during 1 h at 150 V. After, separated proteins were immediately transferred to polyvinylidene difluoride membranes, and the membranes were blocked for 1 h at room temperature using commercial blocking solution (Intercept® Blocking Buffer, LI-COR Biosciences, Inc; Hamburg, The Netherlands). The membranes were then incubated with gentle agitation overnight at 4°C with a rabbit polyclonal anti-NR3C1 antibody (NBP2-42221, Novus Biologicals, Littleton, CO, USA) at dilution 1/400. In addition, a rabbit monoclonal anti-vinculin antibody (926-42215; LI-COR Biosciences, Inc; Hamburg, The Netherlands), at dilution 1/5,000 for 1 h at room temperature, was used as loading control for normalization of results. To visualize Western blot results, membranes were incubated for 1 h at room temperature with an anti-rabbit secondary antibody at 1/10,000 dilution (WesternSure® Goat anti-Rabbit HRP Secondary Antibody, 926-80011; LI-COR Biosciences, Inc; Hamburg, The Netherlands). Finally, the membranes were incubated with a

chemiluminescent substrate mix for 5 min (WesternSure® Premium Chemiluminescent Substrate, 926-95000, LI-COR Biosciences, Inc; Hamburg, The Netherlands) and scanned with C-DiGit™ Blot Scanner (LI-COR Biosciences, Inc; Hamburg, The Netherlands). Western blot band normalization and quantification were performed using Image Studio Lite software version 5.2.5 (LI-COR, Biosciences, Inc; Hamburg, The Netherlands).

RNA EXTRACTION AND cDNA OBTENTION

A commercial miRNeasy Mini Kit (Qiagen, Barcelona, Spain) was used for total RNA extraction from oocytes and cumulus cells, according to manufacturer's instructions. The concentration of RNA was measured using a spectrophotometer (Thermo Scientific™ NanoDrop 2000, Fisher Scientific, Madrid, Spain). The synthesis of the cDNA strand was performed using the commercial High-Capacity RNA-to-cDNA™ Kit (Fisher Scientific, Madrid, Spain), following the manufacturer's instructions. The cDNA samples synthesized were stored at -20°C until further analysis.

QUANTITATIVE REAL-TIME PCR (qPCR)

Quantitative real-time PCR (qPCR) gene expression was performed using a Real-Time PCR Detection System (CFX96™; Bio-Rad Laboratories, Inc; Kabelsketal, Germany) following the steps described elsewhere (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020). The reactions prepared consisted of 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA), 2 µL cDNA, 0.5 µL of each primer, and in a final volume of 10 µL. The PCR protocol setup performed consisted of uracil-DNA glycosylase activation at 50°C for 2 min (1 cycle); denaturation at 95°C for 2 min (1 cycle); denaturation at 95°C for 5 s and

annealing/extension at 60.2°C for 30 s (40 cycles), and a melting curve at 60-95°C (0.5°C increments) for 5 s during each step. Relative quantification of *NR3C1*, *HSD11B1*, *HSD11B2*, *FKBP4*, *FKBP5*, *NR3C2*, *STAT3*, *STAT5A*, *BCL2* and *BAX* mRNA transcripts was performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and *G3PDH* was used as housekeeping gene reference for data normalization. Commercial gene-specific qPCR primers designed for bovine species were used (PrimePCR™ SYBR® Green Assay, Bio-Rad Laboratories, Kabelsketal, Germany). The amplicons of qPCR were loaded into an agarose gel after mixing with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) to confirm the product sizes. The gel was imaged by an imaging system (ChemiDoc XRS+ System, BioRad Laboratories, Inc; Kabelsketal, Germany).

DATA ANALYSES

Statistical analysis of the relative protein and mRNA expression data were analyzed by *R* software version 3.6.1 (*R* Core Team, 2019) with *nlme* (Pinheiro *et al.*, 2021) to develop linear mixed-effects (LME) models and *multcomp* (Hothorn *et al.*, 2008) to perform pairwise comparisons. All data sets were analyzed for normal distribution and homoscedasticity using the Shapiro–Wilk Normality test and Levene's test, respectively. Non-normal distributed data were transformed using the log(x+1) transformation. The threshold of significance was set at $p < 0.05$. For experiment 2, the experimental group and exogenous supplementation of Q10 were included as fixed effects and the replicates as the random part of the LME. For experiment 1, the cortisol treatment, and for experiment 3, the exogenous supplementation of Q10, and the vitrification procedure (vitrified or non-vitrified) were included as fixed effects and the replicates as the random part of the LME. Pairwise comparisons were adjusted by the Sidak test.

RESULTS

EXPERIMENT 1: EFFECT OF CORTISOL DURING IVM ON NR3C1 PROTEIN EXPRESSION AFTER VITRIFICATION

NR3C1 protein relative expression raised significantly after vitrification, especially when cortisol was added during IVM at 0.25 μM (**Figure 1** and **Supplementary Figure 1**).

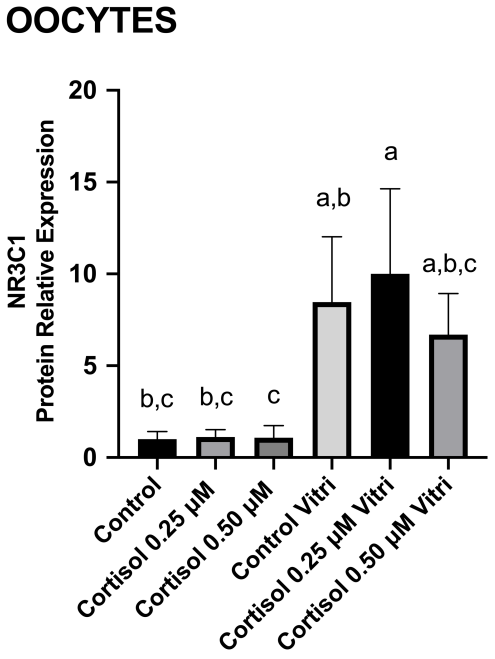

Figure 1. Relative protein expression of NR3C1 after IVM with cortisol supplementation and/or vitrification (Experiment 1). Bar plots of the relative protein expression (fold change) of NR3C1 in oocytes, prior to IVM without exogenous cortisol (Control), with 0.25 μM (Cortisol 0.25 μM), or 0.50 μM (Cortisol 0.50 μM), or after vitrification without cortisol supplementation (Control Vitri), with 0.25 μM (Cortisol 0.25 μM Vitri), or 0.50 μM (Cortisol 0.50 μM Vitri). Incubation conditions during IVM were set at 38.5°C, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between experimental groups.

EXPERIMENT 2: EFFECT OF Q10 SUPPLEMENTATION ON IVM

GC-RELATED GENES

NR3C1 expression raised both in oocytes and cumulus cells after Q10 supplementation during IVM (**Figure 2a** and **3a**), and in cumulus cells after IVM (**Figure 3a**), when compared to oocytes and cumulus cells at germinal vesicle stage. In oocytes, the mRNA expression of *HSD11B1* and *FKBP5* increased after IVM, and Q10 supplementation reduced the mRNA expression to levels comparable to those pre-IVM (**Figure 2b** and 2**e**). In cumulus cells**,** *HSD11B1* and *FKPB5* expression also increased after IVM (**Figure 3b** and 3**e**) and, in the case of *HSD11B1*, decreased after Q10 supplementation, but to higher levels than before IVM (**Figure 3b**). The *FKBP4* mRNA expression in cumulus cells increased after IVM, regardless of the presence of Q10 (**Figure 3d**).The *NR3C2* mRNA expression decreased in oocytes after IVM and Q10 supplemented IVM (**Figure 2f**), while its expression in the cumulus cells increased only after Q10 supplementation (**Figure 3f**). The *STAT5A* mRNA expression in oocytes increased after IVM, and Q10 supplemented IVM (**Figure 2h**). In cumulus cells, it only increased after IVM with Q10 (**Figure 3h**). Moreover, *STAT3* mRNA expression in cumulus cells increased both after IVM, and Q10 supplemented IVM (**Figure 3g**).

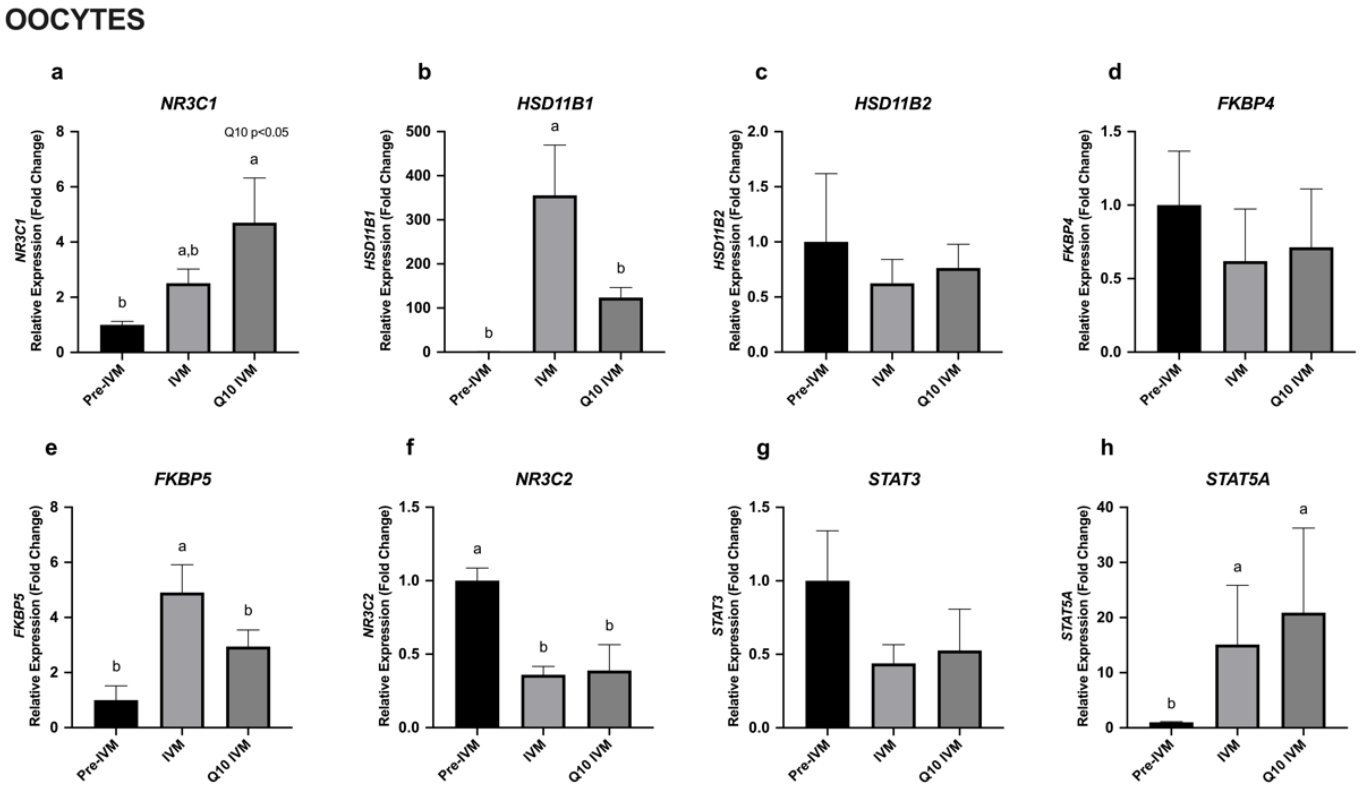

Figure 2. Relative mRNA expression of GC-related genes in oocytes (Experiment 2). Bar plots of the relative mRNA expression (fold change) of *NR3C1* (**a**), *HSD11B1* (**b**), *HSD11B2* (**c**), *FKBP4* (**d**), *FKBP5* (**e**), *NR3C2* (**f**), *STAT3* (**g**), and *STAT5A* (**h**) in oocytes, prior to IVM (Pre-IVM), after IVM (IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 μM). Incubation conditions during IVM were set at 38.5ºC, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental group of oocytes before IVM (Pre-IVM) was established as the reference group. Fixed effect significant factors are included in the graph.

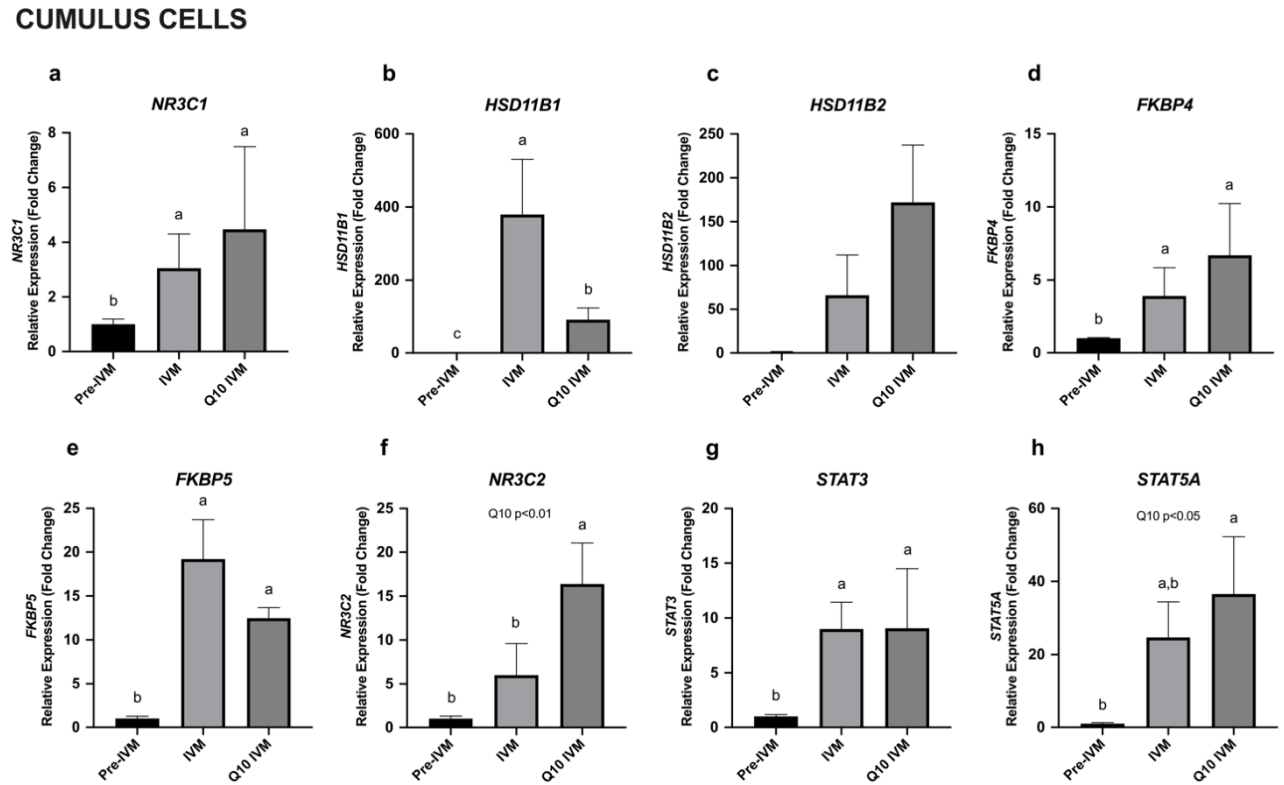

Figure 3. Relative mRNA expression of GC-related genes in cumulus cells (Experiment 2). Bar plots of the relative mRNA expression (fold change) of *NR3C1* (**a**), *HSD11B1* (**b**), *HSD11B2* (**c**), *FKBP4* (**d**), *FKBP5* (**e**), *NR3C2* (**f**), *STAT3* (**g**), and *STAT5A* (**h**) in cumulus cells, prior to IVM (Pre-IVM), after IVM (IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 μM). Incubation conditions during IVM were set at 38.5°C, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental group of cumulus cells before IVM (Pre-IVM) was established as the reference group. Fixed effect significant factors are included in the graph.

APOPTOSIS-RELATED GENES

Q10 increased the anti-apoptotic *BCL2* mRNA expression both in oocytes and cumulus cells (**Figure 4a** and **4d**). In cumulus cells, *BCL2* also increased after IVM, compared to the pre-maturation group (**Figure 4d**). The pro-apoptotic *BAX* mRNA expression increased in both oocytes and cumulus cells after IVM (**Figure 4b** and **4e**), being reduced in oocytes after Q10 supplementation, but to higher levels than before IVM (**Figure 4b**). In addition, the ratio *BAX*:*BCL2* decreased significantly after both IVM and Q10 supplemented IVM when compared to immature oocytes and cumulus cells (**Figure 4c** and 4**f**). The ratio *BAX*:*BCL2* decreased in oocytes after IVM supplemented with Q10 compared to IVM in the absence of Q10 (**Figure 4c**).

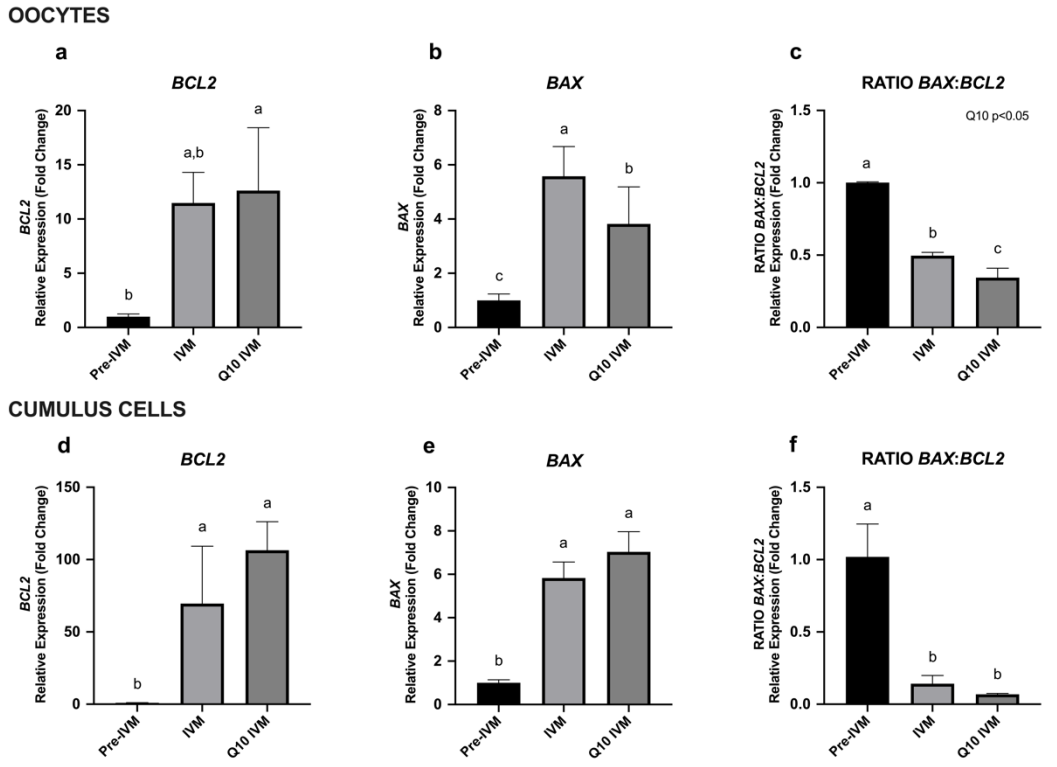

Figure 4. Relative mRNA expression of apoptosis-related genes in oocytes and cumulus cells (Experiment 2). Bar plots of the relative mRNA expression of *BCL2* (**a**,**d**), *BAX* (**b**,**e**), and *BAX*:*BCL2* ratio (**c**, **f**), in oocytes (**a**,**b**,**c**) and cumulus cells (**d**,**e**,**f**), prior to IVM (Pre-IVM), after IVM (IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 μM). Incubation conditions during IVM were set at 38.5°C, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental groups of oocytes (**a**,**b**,**c**) and cumulus cells (**d**,**e**,**f**) before IVM (Pre-IVM) were established as the reference groups, respectively. Fixed effect significant factors are included in the graph.

EXPERIMENT 3: EFFECT OF Q10 SUPPLEMENTATION ON VITRIFICATION

GC-RELATED GENES

Q10 presence reduced the mRNA expression of *HSD11B1* after IVM and vitrification, both in oocytes and cumulus cells (**Figure 5b** and **6b**). In contrast, the presence of Q10 raised the expression of *HSD11B2* mRNA in cumulus cells after

vitrification (Figure 6c). Q10 supplementation during IVM, and also IVM and vitrification, reduced the mRNA expression of *FKBP5* in oocytes and cumulus cells (Figure 5e and 6e).

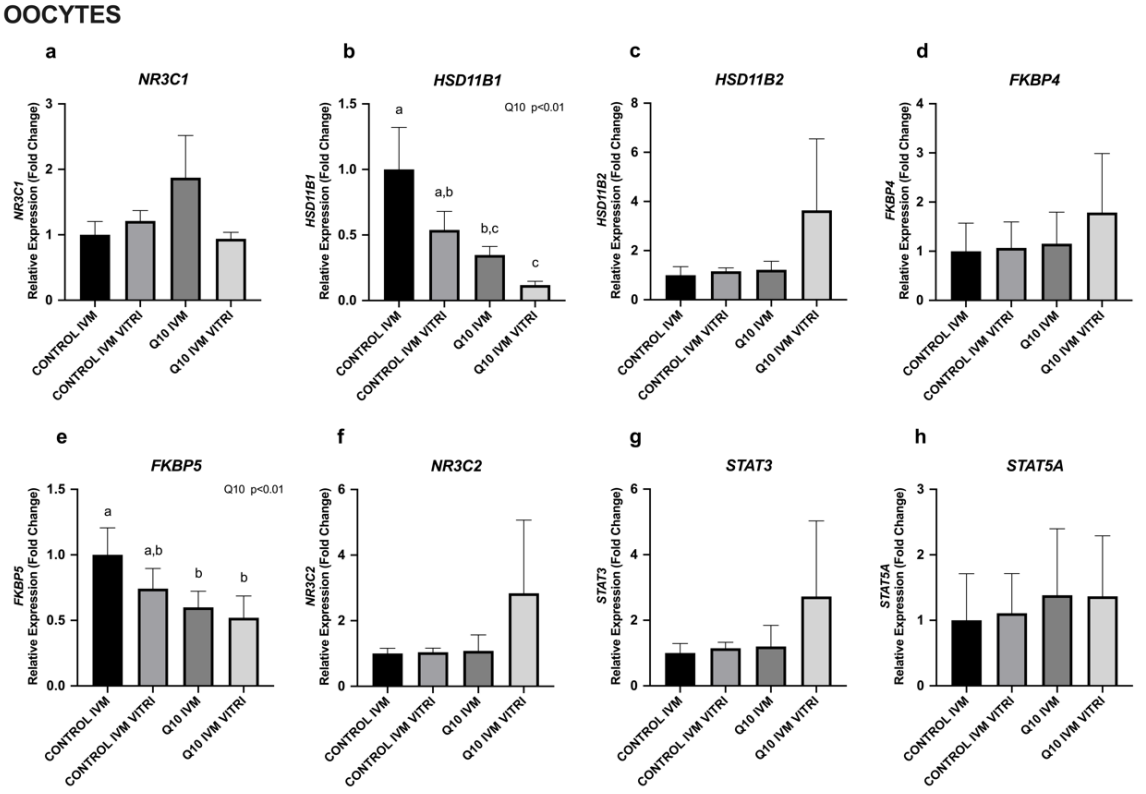

Figure 5. Relative mRNA expression of GC-related genes in oocytes (Experiment 3). Bar plots of the relative mRNA expression (fold change) of *NR3C1* (a), *HSD11B1* (b), *HSD11B2* (c), *FKBP4* (d), *FKBP5* (e), *NR3C2* (f), *STAT3* (g), and *STAT5A* (h) in oocytes, after IVM (Control IVM), after IVM and vitrification at 20 h (Control IVM Vitri), after IVM supplemented with exogenous coenzyme Q10 (50 µM) (Q10 IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 µM) and vitrification at 20 h (Q10 IVM Vitri). Incubation conditions during IVM were set at 38.5ºC, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental group of oocytes after IVM (Control IVM) was established as the reference group. Fixed effect significant factors are included in the graph.

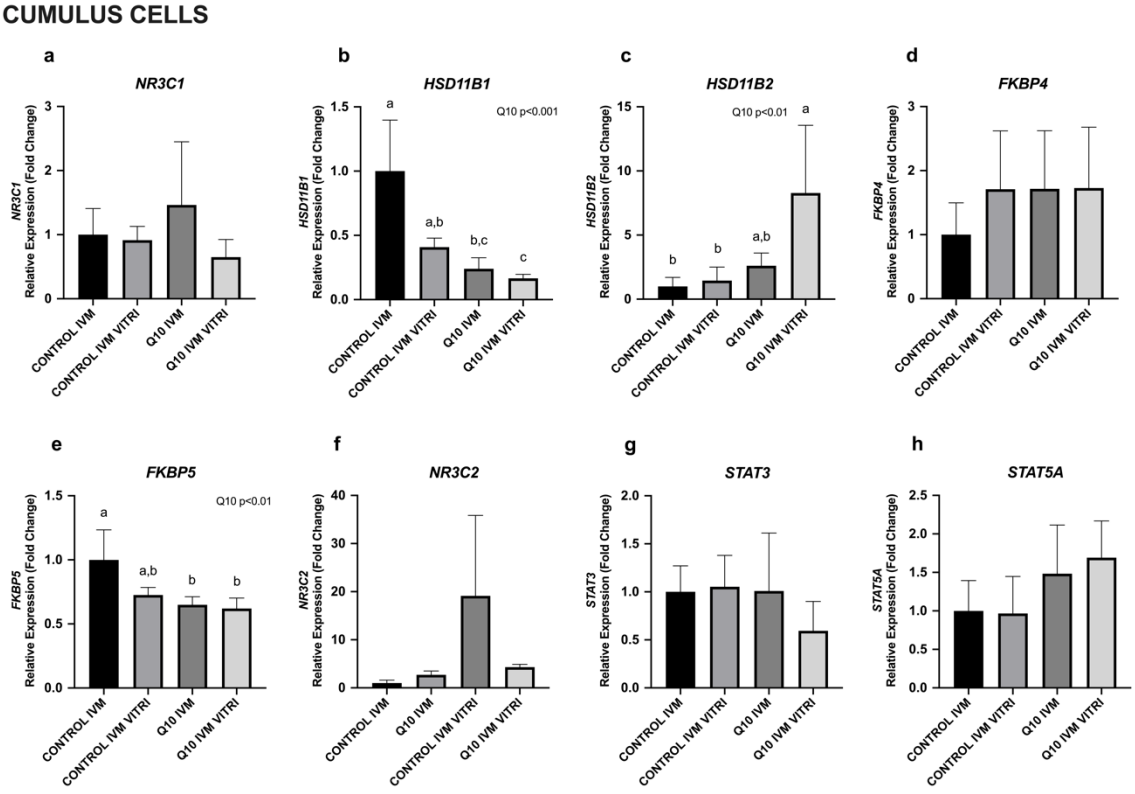

Figure 6. Relative mRNA expression of GC-related genes in cumulus cells (Experiment 3). Bar plots of the relative mRNA expression (fold change) of *NR3C1* (a), *HSD11B1* (b), *HSD11B2* (c), *FKBP4* (d), *FKBP5* (e), *NR3C2* (f), *STAT3* (g), and *STAT5A* (h) in cumulus cells, after IVM (Control IVM), after IVM and vitrification at 20 h (Control IVM Vitri), after IVM supplemented with exogenous coenzyme Q10 (50 μM) (Q10 IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 μM) and vitrification at 20 h (Q10 IVM Vitri). Incubation conditions during IVM were set at 38.5ºC, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental group of cumulus cells after IVM (Control IVM) was established as the reference group. Fixed effect significant factors are included in the graph.

APOPTOSIS-RELATED GENES

In oocytes, the anti-apoptotic *BCL2* mRNA expression increased after vitrification in presence of Q10 compared to the Q10 non-vitrified, the IVM control and IVM control vitrified groups (**Figure 7a**). In contrast, the ratio *BAX*:*BCL2* is reduced after Q10 supplementation compared to the control group (**Figure 7c**). In cumulus cells, the presence of Q10 during IVM in the vitrified group led to an increase in the expression of the anti-apoptotic *BCL2* (**Figure 7d**) when compared to the non-Q10 supplemented vitrified and non-vitrified groups (**Figure 7e**). Moreover, the ratio *BAX*:*BCL2* in cumulus cells decreased significantly after Q10 supplementation during IVM and vitrification, relative to control IVM and control IVM vitrified (**Figure 7f**).

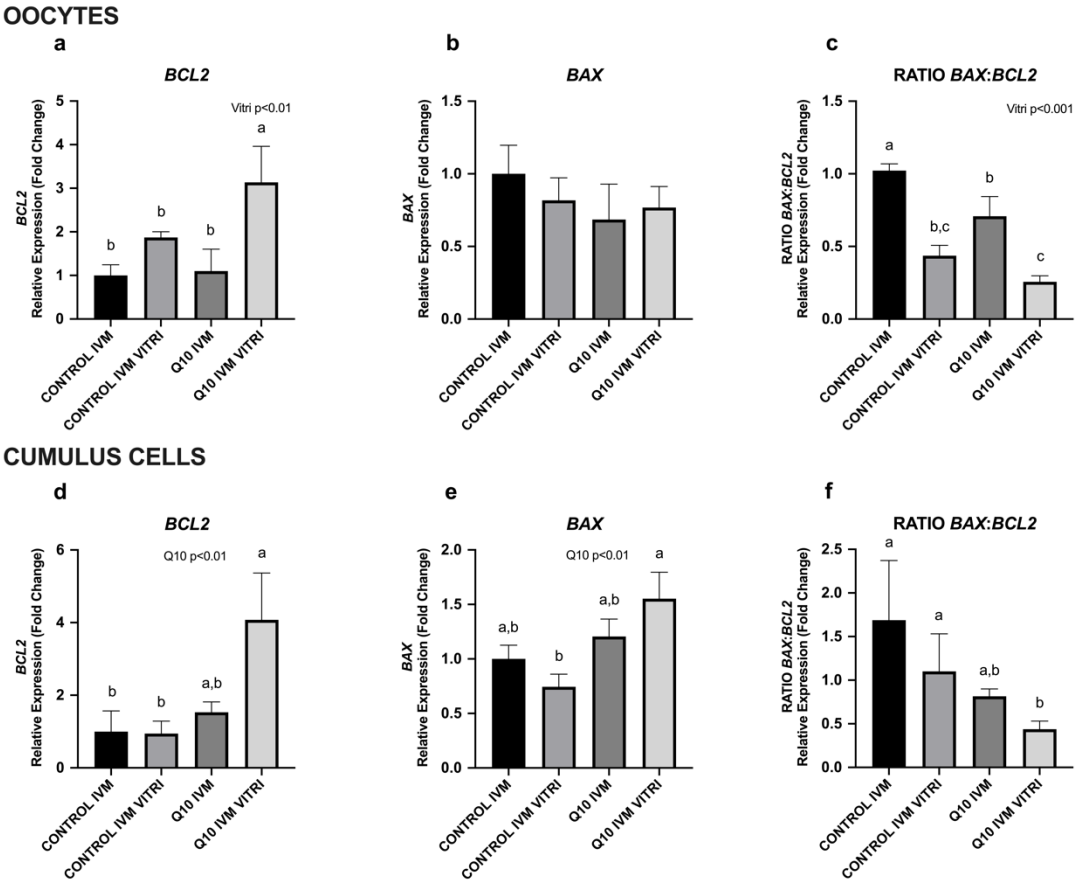

Figure 7. Relative mRNA expression of apoptosis-related genes in oocytes and cumulus cells (Experiment 3). Bar plots of the relative mRNA expression of *BCL2* (**a**,**d**), *BAX* (**b**,**e**), and *BAX*:*BCL2* ratio (**c**, **f**), in oocytes (**a**,**b**,**c**) and cumulus cells (**d**,**e**,**f**), after IVM (Control IVM), after IVM and vitrification at 20 h (Control IVM Vitri), after IVM supplemented with exogenous coenzyme Q10 (50 µM) (Q10 IVM), or after IVM supplemented with exogenous coenzyme Q10 (50 µM) and vitrification at 20 h (Q10 IVM Vitri). Incubation conditions during IVM were set at 38.5°C, 5% CO_2 in humidified atmosphere for 24 h. Different letters represent statistical differences between treatments. The experimental group of oocytes (**a**,**b**,**c**) and cumulus cells (**d**,**e**,**f**) after IVM (Control IVM) were established as the reference groups, respectively. Fixed effect significant factors are included in the graph.

DISCUSSION

Glucocorticoids have demonstrated to play specific roles during the early steps of reproduction (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020; Ruiz-Conca, Gardela, Martínez, *et al.*, 2020; Whirledge & Cidlowski, 2017), being involved in important processes such as gene transcription, energy metabolism and apoptosis (Timmermans *et al.*, 2019; Wang & Harris, 2015). However, specific functions of GC in ovarian physiology and oocyte maturation still remain unclear (Gong *et al.*, 2017; Whirledge & Cidlowski, 2013). In this sense, a better knowledge on glucocorticoid-related (*NR3C1*, *HSD11B1*, *HSD11B2*, *FKBP4*, *FKBP5*, *NR3C2*, *STAT3*, *STAT5A*), and apoptosis-related (*BCL2, BAX*) gene expression during IVM and cryopreservation procedures could be helpful for potentially improving the yield of assisted reproductive techniques in the bovine species.

Despite the scientific advances in recent years, oocyte IVM and vitrification are still suboptimal processes that induce oxidative stress and mitochondrial damage, triggering the activation of apoptotic pathways and impairing oocyte quality (Lord & Aitken, 2013). According to our findings, Q10 supplementation during IVM may be inducing a protective effect against apoptosis in bovine oocytes, reducing the mRNA expression of the pro-apoptotic gene *BAX*, and the pro-apoptotic/anti-apoptotic ratio (*BAX*:*BCL2*). In this regard, Q10 can restore the mitochondrial gene expression of damaged oocytes, and reduce the presence of ROS, acting as a free radical scavenger (Ben-Meir *et al.*, 2015). Moreover, this protective effect of Q10 on apoptosis is present after vitrification, as the expression of the anti-apoptotic *BCL2* mRNA expression was promoted in Q10-supplemented vitrified bovine oocytes and cumulus cells, and the *BAX*:*BCL2* ratio was also decreased in this group. Thus, vitrification may be inducing

mitochondrial-driven apoptosis, which may be partially rescued by the Q10 supplementation, similar to the observed beneficial effects of Q10 on oocyte quality, partially reversing oocyte aging (Zhang *et al.*, 2019).

Glucocorticoids regulate oxidative phosphorylation, gene expression and mitochondrial metabolism (Kokkinopoulou & Moutsatsou, 2021), being crucial for physiological processes which require increased energy, such as lipid metabolism, stress response and reproduction (de Guia & Herzig, 2015; Lapp *et al.*, 2019; Whirledge & Cidlowski, 2010). The unbalance of ROS induced by stressful events can alter GC regulation (Agarwal *et al.*, 2005; Kokkinopoulou & Moutsatsou, 2021), having a negative impact on the oocyte competence. The mechanism of action of GCs over oocyte maturation has remained controversial and notably different across species (da Costa *et al.*, 2016; Scarlet *et al.*, 2017; Yang *et al.*, 1999). While innocuous effects have been shown in equine (Scarlet *et al.*, 2017) and murine (Gong *et al.*, 2017) oocytes, negative effects of GCs have been reported in porcine (Yang *et al.*, 1999), and in ovine oocytes only at high concentrations (250μM) in the latest (González *et al.*, 2010). Those differences regarding GC effects over maturation have been attributed to the presence or absence of two 11β-hydroxysteroid dehydrogenase isoforms, HSD11B1 and HSD11B2, responsible for cortisol activation from cortisone, or cortisol oxidation into inactive cortisone, respectively (Michael *et al.*, 2003; Webb *et al.*, 2008). We found that *HSD11B1* mRNA expression of bovine oocytes and cumulus cells increased during IVM. Beneficial effects on oocyte competence during maturation have been demonstrated after controlled exposure to GCs in bovine (da Costa *et al.*, 2016), and a temporary GC activation by HSD11B1 seems to be relevant for oocyte maturation on this species (Tetsuka & Tanakadate, 2019). However, an excess in GC exposure can also be detrimental for bovine or any other species (Gong *et al.*, 2017). In this sense, the presence of Q10, decreased the expression

of *HSD11B1* mRNA expression, even more when oocytes were vitrified. Additionally, our results showed higher expression of *HSD11B2* mRNA expression after Q10 supplementation and vitrification in cumulus cells. The increased expression of this enzyme, HSD11B2, responsible for inactivating cortisol, has been highlighted as the main reason for the greater resistance against the deleterious effects of GCs in some species. In this regard, murine and equine COCs, less sensitive to GC, showed high expression levels of HSD11B2 (Gong *et al.*, 2017; Scarlet *et al.*, 2017), compared to porcine oocytes, which are considerably more sensitive to GC (Gong *et al.*, 2017; Yang *et al.*, 1999). Moreover, we detected 3-fold higher *HSD11B2* mRNA expression compared to *HSD11B1* in the bovine ampulla tissue during all stages of the estrous cycle (unpublished data). Thus, we hypothesize that Q10, an antioxidant molecule that has already shown positive effects during oocyte vitrification (Hwang *et al.*, 2016; Ruiz-Conca *et al.*, 2017), may induce an increase on *HSD11B2* mRNA expression and a decrease on *HSD11B1* mRNA expression, contributing to the reduction of the GC exposure during a stressful event as vitrification, consequently helping to maintain the GC regulation.

Many of the actions of GCs are mediated by their homonym receptor, the GR/NR3C1 (Rhen & Cidlowski, 2005), which upon binding, is translocated to the nucleus, modulating gene transcription of a great number of pathways, including GC regulation, energy metabolism and apoptosis (Wang & Harris, 2015). The NR3C1 may be crucial for oocyte development, as the receptor presence has been described in oocytes in all follicular stages, except for granulosa and primordial follicles in small ruminants (Pontes *et al.*, 2019). Also, knockout zebrafish for the GC receptor ($nr3c1^{-/-}$) impaired ovulation and oocyte maturation, inhibiting reproduction (Maradonna *et al.*, 2020). Great differences have also been found between species regarding the influence of NR3C1 during oocyte

maturation, as in pig, the NR3C1 may be playing a key role mediating GC effects, while in mouse, those effects may be exerted, at least in part, through an alternative pathway (Gong *et al.*, 2017). To our knowledge, this is the first time that the GC receptor expression levels are described after oocyte vitrification in any species. We found that NR3C1 levels remained similar after cortisol supplementation during IVM, but higher expression was found in oocytes that were vitrified and supplemented with cortisol. Moreover, we also found an increase in the *NR3C1* mRNA expression in oocytes and cumulus cells after Q10 supplementation of IVM. Thus, we hypothesize that an increased expression of NR3C1 receptor may be beneficial for the GC regulatory and transcriptional activity. Also related to the NR3C1 activity is the mineralocorticoid receptor, NR3C2, which is involved in mineralocorticoid signaling, but also has an important ligand affinity for GCs (Baker *et al.*, 2013). We found that *NR3C2* mRNA expression decreased in the oocyte after IVM, and increased in cumulus cells after Q10 supplementation. Recent evidence suggests possible autocrine/paracrine roles of NR3C2 in the bovine follicle (Mukangwa *et al.*, 2020), however, the functional interaction between both receptors may be complex and need further research (Grossmann *et al.*, 2021; Hartmann *et al.*, 2021), as the receptors are evolutionarily related (Baker, 2019).

Furthermore, the immunophilins FKBP5 and FKBP4 are considered relevant in stress signaling and directly involved in the NR3C1 activity (Ratajczak *et al.*, 2015). Acting as cofactors of NR3C1, FKBP4 seems to promote the receptor active translocation towards the nucleus, while FKBP5 acts hindering the NR3C1 translocation and blocking the binding of FKBP4, consequently stopping the NR3C1-mediated transcriptional activity in the nucleus (Wochnik *et al.*, 2005). We found a higher mRNA expression of *FKBP5* and *FKBP4* in cumulus cells after IVM, and also in oocytes in the case of *FKBP5*. Moreover, *FKBP5* mRNA expression was

reduced after Q10 supplementation in oocytes and cumulus cells in vitrified and non-vitrified groups. In this respect, Q10 might help promoting translocation of the receptor to the nucleus by reducing the *FKPB5* inhibitory mechanism of NR3C1, i.e., contributing to the gene transcription and apoptosis regulation. Despite the undeniable relevance of these immunophilins in GC regulation in reproduction (Tranguch *et al.*, 2005; Yang *et al.*, 2006), we are unaware of any previous report of FKBP4/5 expression in oocytes or cumulus cells.

In addition, *STAT3* and *STAT5A*, pleiotropic genes that are relevant during the immune response, are involved in NR3C1 signaling acting as cofactors (Langlais *et al.*, 2012; Petta *et al.*, 2016). We found increased mRNA expression of *STAT5A* in oocytes and *STAT3* in cumulus cells after IVM and Q10 supplemented IVM, while *STAT5A* mRNA expression was increased in cumulus cells after Q10 supplementation. The STAT3 and STAT5A proteins may stimulate the transcriptional activity of NR3C1 by synergism acting as cofactors (de Miguel *et al.*, 2003; Engblom *et al.*, 2007; Petta *et al.*, 2016). Increased *STAT3* mRNA expression in bovine COCs has recently been identified as a potential marker of fertility and necessary for embryo development (Ghanem *et al.*, 2020; Meng *et al.*, 2015). In the case of STAT5A, it has been suggested to play a role during the early embryonic divisions in bovine (Flisikowski *et al.*, 2015) and it is activated in cumulus cells during IVM after IL-6 supplementation, being positive for cumulus expansion (Liu *et al.*, 2009).

CONCLUSIONS

Glucocorticoid regulation gene expression is modulated during oocyte maturation and affected by stressful events, such as vitrification. In this sense, Q10, a powerful endogenous antioxidant involved in the energetic metabolism triggered changes in the mRNA expression of genes related to apoptosis and GC. Supplementation of Q10 during IVM decreased apoptosis, especially after oocyte vitrification. Also, Q10 decreased the mRNA expression of *HSD11B1* in oocytes and increased *HSD11B2* expression in cumulus cells, reducing GC effects after vitrification. Moreover, GC receptor (NR3C1) translocation to the nucleus may be promoted by Q10 supplementation during IVM. *FKBP5* mRNA expression, involved in translocation restriction, was decreased after Q10 supplementation. We also found an increase in the *NR3C1* mRNA expression in oocytes and cumulus cells after Q10 supplementation. Complementarily, cortisol supplementation during vitrification caused a higher expression of the NR3C1 receptor protein. Further mechanistic studies focused on the specific role of each molecule involved in GC regulation could be helpful for future potential applications in reproductive technologies.

Declaration of interest: None.

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Author contributions: Mateo Ruiz-Conca: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing - original draft. Jaume Gardela: Methodology; Software; Data curation; Writing - review and editing. Teresa Mogas: Conceptualization; Funding acquisition; Project administration; Writing - review and editing. Manel López-Béjar: Conceptualization; Supervision; Funding acquisition; Project administration; Writing - review and editing. Manuel Álvarez-Rodríguez: Conceptualization; Supervision; Funding acquisition; Project administration; Writing - review and editing.

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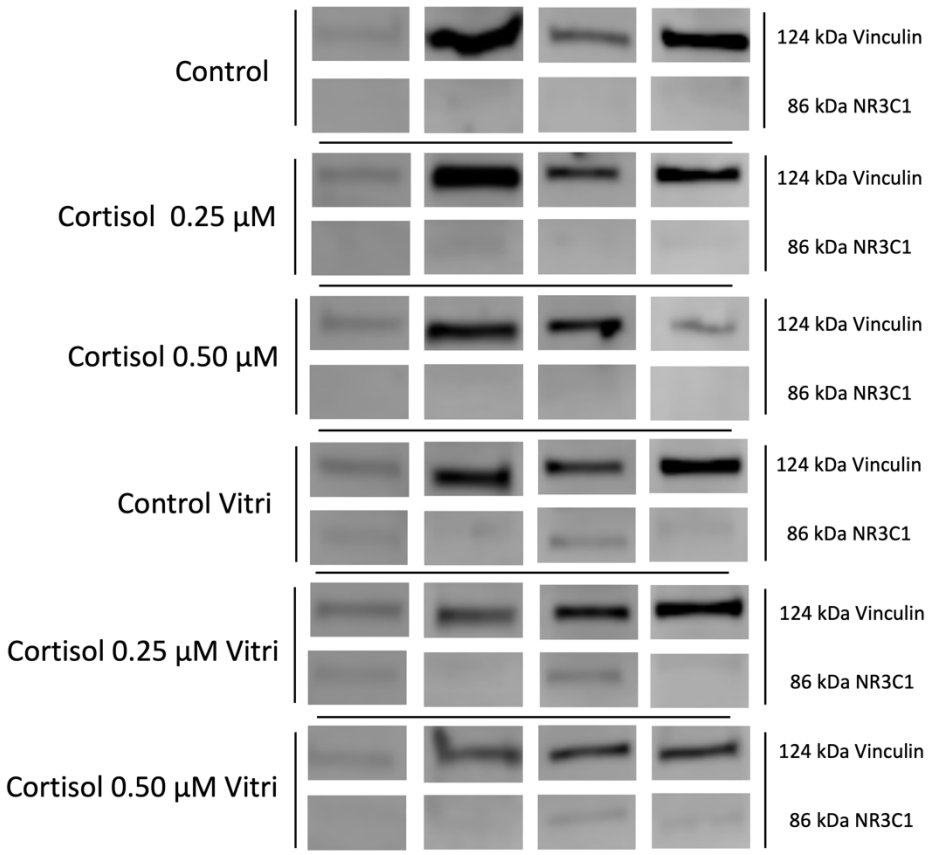
SUPPLEMENTARY FIGURES



Supplementary Figure 1. NR3C1 and vinculin protein expression analyzed by Western blot in oocytes after IVM including cortisol supplementation and/or vitrification (Experiment 1). Bands at 86 kDa show the protein expression of NR3C1, and bands at 124 kDa show the protein expression of vinculin, used as endogenous protein control. The experimental groups included IVM without exogenous cortisol (Control), IVM with 0.25 µM (Cortisol 0.25 µM), or IVM with 0.50 µM (Cortisol 0.50 µM), or after vitrification without cortisol supplementation (Control Vitri), with 0.25 µM (Cortisol 0.25 µM Vitri), or 0.50 µM (Cortisol 0.50 µM Vitri). Incubation conditions during IVM were set at 38.5°C, 5% CO_2 in humidified atmosphere for 24 h.

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CHAPTER III

NR3C1 and Glucocorticoid-Regulatory Genes mRNA and Protein Expression in the Endometrium and Ampulla during the Bovine Estrous Cycle

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ABSTRACT

The bovine reproductive tract exhibits changes during the estrous cycle modulated by the interplay of steroid hormones. Glucocorticoids can be detrimental when stress-induced but are relevant at baseline levels for appropriate reproductive function. Here, an analysis of quantitative real-time PCR was performed to study the bovine glucocorticoid-related baseline gene transcription in endometrial and ampullar tissue samples derived from three time points of the estrous cycle, stage I (Days 1-4), stage III (Days 11-17) and stage IV (Days 18-20). Our results revealed expression differences during stages, as expression observed in the ampulla was higher during the post-ovulatory phase (stage I), including the glucocorticoid receptor *NR3C1*, and some of its regulators, involved in glucocorticoid availability (*HSD11B1* and *HSD11B2*) and transcriptional actions (*FKBP4* and *FKBP5*). In contrast, in the endometrium, higher expression of the steroid receptors was observed during the late luteal phase (stage III), including *ESR1*, *ESR2*, *PGRMC1* and *PGRMC2,* and *HSD11B1* expression decreased, while *HSD11B2* increased. Moreover, at protein level, FKBP4 was higher expressed during the late luteal phase, and NR3C1 during the pre-ovulatory phase (stage IV). These results suggest that tight regulation of the glucocorticoid activity is promoted in the ampulla, when reproductive events are taking place, including oocyte maturation. Moreover, most expression changes in the endometrium were observed during the late luteal phase, and may be related to the embryonic maternal recognition. In conclusion, the glucocorticoid regulation changes across the estrous cycle and may be playing a role on the reproductive events occurring in the bovine ampulla and endometrium.

Keywords: Glucocorticoid; cow; cortisol; steroid receptors; qPCR; western blot.

INTRODUCTION

Many of the physiological vertebrate functions, including metabolism, behavior, immunity, development, stress response, and reproduction, are regulated by steroid hormones (Wang & Harris, 2015). Some of these functions are exerted through the nuclear receptor's family, a diverse group of transcription factors that have steroids as ligands (Carson-Jurica *et al.*, 1990). These receptors may have contributed to the evolution of multicellular animals, assumed to be helped by their diversification into different steroid receptors (Baker, 2019). Evolutive changes in the primordial receptor may have happen in order to provide a specialized hormonal response to the more complex and diverse physiological functions present in vertebrates (Baker *et al.*, 2015; Bertrand *et al.*, 2004). In reproduction, adrenal and gonadal steroids are known to regulate the physiological changes occurring in the female reproductive tract during the sexual cycle (Barton *et al.*, 2020; Whirledge & Cidlowski, 2017). As in other animal species, in bovine, great dynamic modifications of the reproductive tract environment are taking place during the different stages of the estrous cycle (Ireland *et al.*, 1980), both in the oviductal and uterine portions (Binelli *et al.*, 2018; Forde & Lonergan, 2012). Although there are extensive studies focusing on gametes transport through the female tract and the interactions occurring between the maternal environment and spermatozoa and oocytes (Hunter, 2012; Talevi & Gualtieri, 2010), information about oviductal and endometrial regulation by steroids is especially scarce in the case of glucocorticoids (GCs) (Binelli *et al.*, 2018).

The complex mechanisms underlying the required balance in the GC action have recently started to be deemed of much importance for reproduction (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020; Whirledge & Cidlowski, 2017; Whirledge *et al.*, 2015). While sustained stress-induced levels of GCs are often detrimental for fertility (Pontes *et al.*, 2019), baseline GC levels are necessary for an adequate physiological and reproductive function (Whirledge & Cidlowski, 2017). In comparative studies, data suggest that evolutionary changes in response to hormones occur more often by producing changes in the receptor, enzymes and co-regulators, rather than altering the hormone signal (Adkins-Regan, 2013). Many factors influence the extent of the GC action, including spatiotemporal patterns of GC exposure, which seems to variate greatly, even among closely related species (Lattin *et al.*, 2015). Moreover, the fine-tuning of the GC regulation appears to be determined in a tissue-specific manner (Lattin *et al.*, 2015; Martins & de Castro, 2021), displaying variations between anatomical regions of the reproductive tract (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020; Ruiz-Conca, Gardela, Martínez, *et al.*, 2020; Whirledge & DeFranco, 2018). In humans, GC synthetic analogues are often prescribed as fertility treatments for reducing inflammatory responses when impaired endometrial receptivity is present (Robertson *et al.*, 2016). In contrast, exposure to sustained stress-induced GC levels seem to inhibit endometrial receptivity (Park *et al.*, 2021). Thus, these specific responses suggest the need for a necessary balance in the GC levels to differentiate between pathogenicity and reproductive physiology.

Indeed, GCs have been proposed to play a key role in the reproduction of mammals, mainly through the glucocorticoid receptor (NR3C1). The role of NR3C1 has been highlighted in both female and male reproduction on different species, where, respectively, uterine presence of the receptor is crucial for implantation in mice (Whirledge *et al.*, 2015), and relevant for testicular function

in humans (Nordkap *et al.*, 2017). The GC availability to NR3C1 seems to be regulated by two 11β-hydroxysteroid dehydrogenases (HSD11B1 and HSD11B2), responsible for the conversion of cortisone into active cortisol, and the inactivation of cortisol to cortisone, respectively (Michael *et al.*, 2003). Moreover, the receptor's biological mechanism of action is complex, involving a great number of co-factors and repressors, including the FK506-binding immunophilins FKBP4 and FKBP5 (Petta *et al.*, 2016; Ratajczak *et al.*, 2015; Wochnik *et al.*, 2005; Zannas *et al.*, 2019). In the absence of ligand, NR3C1 remains inactive in the cytoplasm and bound to the immunophilin FKBP5, causing no genomic effect. Nevertheless, when GCs are available, the immunophilin FKBP5 is interchanged by the FKBP4, promoting the translocation of the multimeric receptor's complex to the nucleus, promoting active transcription changes either by stimulating or repressing gene expression (Wang & Harris, 2015). On the other hand, the oocyte and embryo sensitivity to GC seems to differ substantially between species. In bovine, a regulated local GC environment seems to be necessary for the reproductive physiology of this species (Acosta *et al.*, 2005; da Costa *et al.*, 2016; Tetsuka & Tanakadate, 2019), although there is a lack of studies that delve into the biological mechanisms behind these findings. In addition, other factors may be involved in the GC actions, including the STAT proteins. The STAT3 and STAT5A have demonstrated roles in transcriptional actions in interaction with NR3C1 (Langlais *et al.*, 2012; Petta *et al.*, 2016), including the regulation of TLR2, involved in the immune actions of the reproductive tract tissues (Ezz *et al.*, 2019). Also, the NR3C1 is closely related to other steroid receptors, such as the mineralocorticoid receptor (NR3C2), the androgen receptor (AR), the progesterone receptors components (PGRMC1 and PGRMC2) and the estrogen receptors (ESR1 and ESR2), all of them having roles in the sex steroid hormone levels during the reproductive cycle (Gibson *et al.*, 2020; Lozovyy *et al.*, 2021; Mukangwa *et al.*, 2020).

Our objective is to describe the GC-related expression changes present in the bovine ampulla and uterus across the stages of the estrous cycle. We hypothesize that the expression of steroid receptors, including *NR3C1* and related genes, is modulated across the estrous cycle, conditioning the physiological dynamic modifications at tissue level occurring in the bovine female reproductive tract. Therefore, GC signaling may influence basal-related changes of the reproductive environment, which might be also relevant for later reproductive events taking place in the oviduct and uterus. For this purpose, we analyzed the mRNA and protein expression changes observed in the ampulla and the endometrium during the pre-ovulatory, post-ovulatory and late luteal phases (stage IV, I and III, respectively) of the bovine estrous cycle. We assessed the mRNA and protein expression of the glucocorticoid receptor gene (*NR3C1)* and the FK506-binding immunophilins (*FKBP4*, *FKBP5)*, directly involved in receptor translocation, activation and repression; the mRNA expression of 11β-hydroxysteroid dehydrogenases, involved in GC ligand availability (*HSD11B1*, *HSD11B2)*; signal transducers and activators of transcription 3 and 5A (*STAT3*, *STAT5A)*, involved in the glucocorticoid receptor signaling and function; the toll-like receptor 2 (*TLR2)*; and steroid receptors, including the mineralocorticoid receptor (*NR3C2)*, membrane-associated progesterone receptor component 1 and 2 (*PGRMC1*, *PGRMC2)*, androgen receptor (*AR)*, and estrogen receptor 1 and 2 (*ESR1*, *ESR2)*.

MATERIALS AND METHODS

TISSUE SAMPLES COLLECTION

The tissue samples were obtained from crossbreed beef post-pubertal heifers (*Bos taurus*) (*n* = 20) sacrificed in a local slaughterhouse (Escorxador de Sabadell, Barcelona, Spain) for commercial purposes. Animals were classified according to the morphological classification established by Ireland *et al*. (Ireland *et al.*, 1980) for the different stages of the bovine estrous cycle: stage I (post-ovulatory phase), from Day 1 to 4 (*n* = 6); stage II (early luteal phase), from Day 5 to 10 (not included due to low number of animals); stage III (late luteal phase), from Day 11 to 17 (*n* = 8); stage IV (pre-ovulatory phase), from Day 18 to 20 (*n* = 6). Five tissue fragments (5 mm × 5 mm, each) from the endometrium and ampulla, were obtained by dissection of the reproductive tract. The tissue samples were obtained ipsilateral to the antral follicle or corpus luteum from whole sections of the ampulla and endometrium tissue fragments obtained from intercaruncular areas at the uterine horn base. Folicullar fluid was obtained by aspiration of antral follicles. All samples were processed in less than 1 h and stored at -80ºC in RNAlater ™ (Fisher Scientific, Gothenburg, Sweden), until RNA and protein isolation.

RNA ISOLATION AND MEASUREMENT

The total RNA extraction was performed using a TRIzol-based protocol previously described (Ruiz-Conca, Gardela, Jauregi-Miguel, *et al.*, 2020). In summary, 1 mL TRIzol was added to five tissue fragments (1 mm x 1 mm, each; 80 mg) of each individual sample from the endometrium and the ampulla of every animal included in the study (*n* = 20) before being disrupted mechanically and

homogenized (TissueLyser II with 7 mm stainless steel beads; Qiagen, Sollentuna, Sweden). The homogenized tissues were centrifuged at 12000 X *g* for 10 min at 4°C. Phase separation was conducted by adding 1-Bromo-3-chloropropane and shaking thoroughly before centrifugation (12000 X *g*, 15 min, 4°C). Then, 2-propanol and RNA precipitation solution (1.2 M NaCl and 0.8 M $Na_2C_6H_6O_7$) was added and centrifuged (12000 X *g*, 10 min, 4°C). Also, 1 mL of 75% ethanol was added to each sample prior to centrifugation (7500 X *g*, 5 min, 4°C). The RNA pellet was obtained after supernatant discard and 30 min dry in the fume hood. Finally, RNA was diluted in 30 µL of RNase free water.

Subsequently to 30 min on ice after isolation, RNA concentration was assessed spectrophotometrically by measuring the absorbance at 260 nm (NanoDrop™ 2000, Fisher Scientific, Gothenburg, Sweden). The RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only samples with RNA integrity values (RIN) higher than 8.0 were used (Schroeder *et al.*, 2006). The RNA 260/230 and 260/280 absorbance ratios, and the RIN values for each sample are shown in **Supplementary Table 1**. The synthesis of the cDNA first strand was completed by using the High-Capacity RNA-to-cDNA™ Kit (Fisher Scientific, Gothenburg, Sweden) following the manufacturer's indications. A total of 5 µg RNA was mixed in a final volume of 50 µL for the reverse transcription reaction. All cDNA samples were kept at −20°C until further analyses.

QUANTITATIVE REAL-TIME PCR (qPCR)

Quantitative real-time PCR (qPCR) gene expression was performed using a Real-Time PCR Detection System (CFX96™; Bio-Rad Laboratories, Inc; Kabelsketal, Germany) following the steps described elsewhere (Ruiz-Conca, Gardela, Jauregi-

Miguel, *et al.*, 2020). The mRNA relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method for relative quantification described elsewhere (Livak & Schmittgen, 2001). The reactions prepared consisted of 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA), 2 µL cDNA, 0.5 µL of each primer, and in a final volume of 10 µL. The PCR protocol setup performed consisted of uracil-DNA glycosylase activation at 50°C for 2 min (1 cycle); denaturation at 95°C for 2 min (1 cycle); denaturation at 95°C for 5 s and annealing/extension at 60.2°C for 30 s (40 cycles), and a melting curve at 60-95°C (0.5°C increments) for 5 s during each step. Fifteen commercial gene-specific qPCR primers for bovine were used (*G3PDH, NR3C1, FKBP4, FKBP5, HSD11B1, HSD11B2, NR3C2, AR, ESR1, ESR2, PGRMC1, PGRMC2, TLR2, STAT3, STAT5A*; PrimePCR™ SYBR® Green Assay, Bovine; Bio-Rad Laboratories, Inc; Kabelsketal, Germany). Preliminary, four different housekeeping genes were used for cDNA data normalization (*G3PDH, β-ACTIN, HPRT1, TBP*). The glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene was chosen as the housekeeping gene used for normalization due to the most constant expression showed in the tissue samples included in the study. Two technical replicates were performed for each sample and each primer used. Specific primer sequences belong to the company (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Chromosomal location of the primers and amplicon lengths are shown in **Table 1.** The amplicons of qPCR were loaded into an agarose gel after mixing with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) to confirm the product sizes. The gel was imaged by an imaging system (ChemiDoc XRS+ System, BioRad Laboratories, Inc; Kabelsketal, Germany).

Table 1. Primers used for gene expression qPCR analysis.

Gene	Chromosome location	Product size (bp)	Splice variants targeted
G3PDH	5:104239749-104241657	92	ENSBTAT00000037753
NR3C1	7:56236814-56247471	85	ENSBTAT00000025941
FKBP4	5:107449938-107450858	119	ENSBTAT00000009998
FKBP5	23:9522465-9522614	120	ENSBTAT00000064387
HSD11B2	18:35163620-35163719	70	ENSBTAT00000007470
HSD11B1	16:75465890-75506713	120	ENSBTAT00000020078
NR3C2	17:9743910-9953168	109	ENSBTAT00000003291
ESR1	9:90220286-90250637	112	ENSBTAT00000009422
ESR2	10:76757251-76757370	90	ENSBTAT00000005899
PGRMC1	X:3476798-3476910	83	ENSBTAT00000026053
PGRMC2	17:29888146-29889663	81	ENSBTAT00000014390
AR	X:88418395-88425354	116	ENSBTAT00000030067
TLR2	17:3950948-3951081	104	ENSBTAT00000010530
STAT3	19:43063874-43065290	114	ENSBTAT00000028687
STAT5A	19:43047935-43048136	65	ENSBTAT00000034831

bp: base pair; *G3PDH*: glyceraldehyde-3-phosphate dehydrogenase; *NR3C1*: nuclear receptor subfamily 3 group C member 1; *FKBP4*: FK506-binding prolyl isomerase 4; *FKBP5*: FK506-binding prolyl isomerase 5; *HSD11B1*: hydroxysteroid 11-beta dehydrogenase 1; *HSD11B2*: hydroxysteroid 11-beta dehydrogenase 2; *NR3C2*: nuclear receptor subfamily 3 group C member 2; *ESR1*: estrogen receptor 1; *ESR2*: estrogen receptor 2; *PGRMC1*: progesterone receptor membrane component 1; *PGRMC2*: progesterone receptor membrane component 2; *AR*: androgen receptor; *STAT3*: signal transducer and activator of transcription 3; *STAT5A*: signal transducer and activator of transcription 5A.

PROTEIN EXTRACTION AND WESTERN BLOT FOR FKBP4 AND FKBP5

Three tissue fragments (1 mm × 1 mm) from each ampulla and endometrium individual sample of every animal included in the study (*n* = 20) were plunged into a mix of RIPA lysis buffer (Fisher Scientific, Gothenburg, Sweden) containing protease inhibitor and EDTA (Ethylenediaminetetraacetic acid; Thermo Scientific™ Halt™ Proteinase Inhibitor Cocktail (100X); Fisher Scientific, Gothenburg, Sweden). Samples were then homogenized (10 s vortex every 15 min) for 1 h while maintained in ice, prior to centrifugation at 13000 X g, 10 min, 4°C for protein separation. The concentration of proteins was assessed by using a commercial colorimetric protein assay (DC Protein Assay; Bio-Rad Laboratories, Inc; Kabelsketal, Germany). For Western blot preparation of each sample, 25 µg of protein were mixed with sample buffer (4x NuPAGE LDS; Fisher Scientific, Gothenburg, Sweden) and dithiothreitol (500 mM), and heated at 70°C for 10 min. Denatured proteins and molecular marker (Odyssey® One-Color Protein Molecular Weight Marker; LI-COR Biosciences, Inc; Hamburg, The Netherlands) were then loaded into an 12-15% SDS-polyacrylamide gel and run for electrophoresis (1 h 15 min, 150 V). Separated proteins in gel were transferred (1 h, 100 V) into an activated 0.2 µm polyvinylidene difluoride membrane (PVDF membrane; Fisher Scientific, Gothenburg, Sweden). The membranes were then blocked at room temperature for 1 h (Intercept™ Tris-buffered saline blocking buffer, LI-COR Biosciences, Inc; Hamburg, The Netherlands), and incubated with rabbit polyclonal antibody anti-FKBP52 (FKBP4) at 1:500 dilution (ab97306; Abcam, Cambridge, UK) or rabbit monoclonal antibody anti-FKBP51 (FKBP5) at 1:1000 dilution (ab126715; Abcam, Cambridge, UK) at room temperature for 2 h. Additionally to the primary antibodies against target proteins, rabbit monoclonal antibody anti-vinculin at 1:5000 dilution (926-42215; LI-COR Biosciences, Inc; Hamburg, The Netherlands) for 1 h at room temperature, was used as loading

control for Western blot normalization. After membranes were incubated for 1 h at room temperature with goat anti-rabbit secondary antibody at 1:10000 dilution (WesternSure® Goat anti-Rabbit HRP Secondary Antibody, 926-80011; LI-COR Biosciences, Inc; Hamburg, The Netherlands). Finally, the membranes were incubated with chemiluminescent substrate mix for 5 min (WesternSure® PREMIUM Chemiluminescent Substrate, 926-95000, LI-COR Biosciences, Inc; Hamburg, The Netherlands) and scanned with C-DiGit™ Blot Scanner (LI-COR Biosciences, Inc; Hamburg, The Netherlands). Western blot bands normalization and quantification was performed by using Image Studio Lite software version 5.2.5 (LI-COR, Biosciences, Inc; Hamburg, The Netherlands).

COMPETITIVE ELISA FOR NR3C1PROTEIN QUANTITATIVE DETERMINATION

Competitive ELISA (Bovine Glucocorticoid Receptor (NR3C1) ELISA Kit; MBS7263720; MyBiosource; San Diego, CA, USA) was used for quantitative determination of NR3C1 protein abundance. Briefly, three tissue fragments (1 mm × 1 mm, each; 30-50 mg) from each sample were weighted and homogenized (10 s vortex every 15 min) in 250 µL of a mix of RIPA lysis buffer (Fisher Scientific, Gothenburg, Sweden) containing protease inhibitor and EDTA (Ethylenediaminetetraacetic acid; Thermo Scientific™ Halt™ Proteinase Inhibitor Cocktail (100X); Fisher Scientific, Gothenburg, Sweden) and centrifugated at 13000 X *g* for 15 min, 4°C. Then, 100 µL of protein extracts from each sample were pipetted into the wells of a pre-coated plate, and 100 µL of balance solution and 50 µL of enzyme conjugate (except the blank) were added. The plate was incubated in the dark for 1 h at 37°C. After incubation, the wells were decanted and washed prior to be incubated with a substrate for the HRP enzyme. Finally, after 15 min at 37°C, an acid solution was added to stop the enzyme-substrate

reaction. The absorbance values were spectrophotometrically measured at 450 nm using a microplate reader (Sunrise-147 Basic Tecan; Tecan Austria GmbH, Grödig, Austria). The NR3C1 concentration of each sample was interpolated from a standard curve. This standard curve was plotted using the absorbance measured in different dilutions of standard samples of known concentrations within the detection range of the kit. Moreover, a weight-normalization was performed to express the concentration of NR3C1 protein per total concentration of protein extracted. All samples and standards were assayed in duplicate. The precision within the test was assessed by calculating coefficients of variation (CV, where CV = SD/mean × 100) from duplicate samples. The CV for NR3C1 was 3.23% and a sensitivity of 0.1 ng/mL was obtained. No significant cross-reactivity or interference between NR3C1 and analogues has been described by the kit manufacturer.

PROGESTERONE AND ESTRADIOL MEASUREMENT IN FOLLICULAR FLUID

Follicular fluid was obtained by aspiration of healthy antral follicles visible in the ovarian cortex using an 18 G needle with a 10 mL syringe. After collection, follicular fluid samples were centrifuged at 5000 X *g* for 10 min and stored at -20ºC until being analyzed. Progesterone (P4) and estradiol (E2) levels on follicular fluid were measured by means of enzyme immunoassay (EIA) following protocols previously described by our group (Maya-Soriano *et al.*, 2013). Commercial kits were used for hormonal determination (Progesterone ELISA KIT and Estradiol ELISA KIT; Neogen Corporation, Ayr, UK; estimated sensitivity of 0.23 ng P4/mL and 0.012 ng E2/mL). Manufacturer's indications estimated that cross-reactivity (>0.02%) of the commercial P4 antibody with other steroid hormones was 2.5% for deoxycorticosterone, 2.0% for corticosterone, 2.0% for pregnenolone, 1.0%

for 4-androstenedione, 0.4% for 17-hydroxyprogesterone, 0.29% for testosterone, 0.2% for cortisol, 0.2% for cortisone, 0.2% for dehydroepiandrosterone, 0.2% for E2, and 0.2% for estrone. Manufacturer's indications estimated that cross-reactivity (>0.02%) of the commercial E2 antibody with other steroid hormones was 1.0% for testosterone, 0.41% for estriol, and 0.1% for estrone.

STATISTICAL ANALYSIS

The software CFX Maestro™ 1.1 version 4.1.2433.1219 (Bio-Rad Laboratories, Inc; Kabelsketal, Germany) was used for the complete genetic data analysis and Image Studio software version 5.2.5 (LI-COR, Biosciences, Inc; Hamburg, The Netherlands) was used for the Western blot analyses. The statistical analysis was performed in *R* software version 3.6.1 (R Core Team, 2019). Normal distribution and homoscedasticity were checked using the Shapiro–Wilk Normality test and Levene's test, while log(x+1) was used for data transformation into normal distribution. The packages *nlme* (Pinheiro & Bates, 2011) and *multcomp* (Hothorn *et al.*, 2008) were used to perform linear mixed effects models (LME), and to conduct pairwise comparisons adjusted by Tukey's test, respectively. The estrous cycle stages (stage I, III and IV) were considered as the fixed factor of the LME, and the samples were included in the random part of the LME. Stage I (post-ovulatory phase) was set as an arbitrary reference for relative gene expression. Comparisons between specific pairs of genes (*HSD11B1*/*HSD11B2* and *FKBP4*/*FKBP5*) was conducted using T-test when data showed normal distribution, and Mann-Whitney U test for non-normal data. HSD11B1 and FKBP4, respectively, were set as arbitrary references for relative gene expression of each specific pair of genes. Data are presented as mean ± standard error of the mean

(SEM). The significance threshold was established at $p < 0.05$. Correlation of the qPCR expression data was analyzed using multiple Spearman's rank correlation coefficient to find the connection between the relative fold change of each studied gene, and also with the follicular fluid concentrations of E2 and P4. Relative protein expression analyses of NR3C1, FKBP4 and FKBP5 were conducted by performing one-way ANOVA test, followed by Tukey's multiple comparisons test. Data are presented as mean ± SEM. The significance threshold was established at $p < 0.05$. Additionally, the correlations between relative mRNA levels and relative protein expression of each sample were calculated using multiple Spearman's rank correlation coefficient.

RESULTS

AMPULLARY AND ENDOMETRIAL mRNA EXPRESSION IN THE PRE-OVULATORY PHASE vs. POST-OVULATORY PHASE (STAGE IV – STAGE I)

The ampullary mRNA expression levels of *NR3C1*, *ESR1*, and *AR* were significantly higher ($p < 0.05$) in stage I, compared to stage IV (**Figure 1**). Regarding endometrium, we found that the mRNA expression of *HSD11B2* was significantly lower ($p < 0.05$) in stage I than in stage IV (**Figure 2**).

AMPULLARY AND ENDOMETRIAL mRNA EXPRESSION IN THE POST-OVULATORY PHASE vs. LATE LUTEAL PHASE (STAGE I – STAGE III)

The ampullary mRNA expression level of *NR3C2* in stage III was significantly higher ($p < 0.05$) in comparison to stage I. Also, mRNA expression level of *NR3C1*, *FKBP4*, *FKBP5*, *HSD11B1*, *HSD11B2*, *ESR1*, *AR*, and *PGRMC2* ($p < 0.05$) was

higher in stage I, than in stage III in the ampulla (**Figure 1**). In the endometrium, the mRNA expression level of *FKBP4, NR3C2, HSD11B2, ESR1, ESR2, PGRMC1, PGRMC2, TLR2,* and *STAT3* in stage III, was significantly higher ($p < 0.05$) in comparison to stage I. Also, mRNA *HSD11B1* expression observed in stage III was significantly lower ($p < 0.05$) than in stage I in the endometrium (**Figure 2**).

AMPULLARY AND ENDOMETRIAL mRNA EXPRESSION IN LATE LUTEAL PHASE vs. PRE-OVULATORY PHASE (STAGE III – STAGE IV)

In the ampulla, during stage IV, we observed higher mRNA expression level of *NR3C1*, *FKBP4*, *FKBP5*, *ESR1*, *AR,* and *PGRMC2* ($p < 0.05$) compared to stage III. In the same tissue, we found in stage IV lower ($p < 0.05$) mRNA expression of *NR3C2*, *TLR2,* and *STAT5A,* when compared to late diestrus (stage III) (**Figure 1**). In the endometrium, higher mRNA expression level of *HSD11B1* in stage IV was described, when compared to stage III ($p < 0.05$). Also, our results show lower ($p < 0.05$) mRNA expression of *NR3C2*, *HSD11B2*, *ESR1*, *ESR2*, *PGRMC1,* and *STAT3* in stage IV compared to stage III in the endometrium (**Figure 2**).

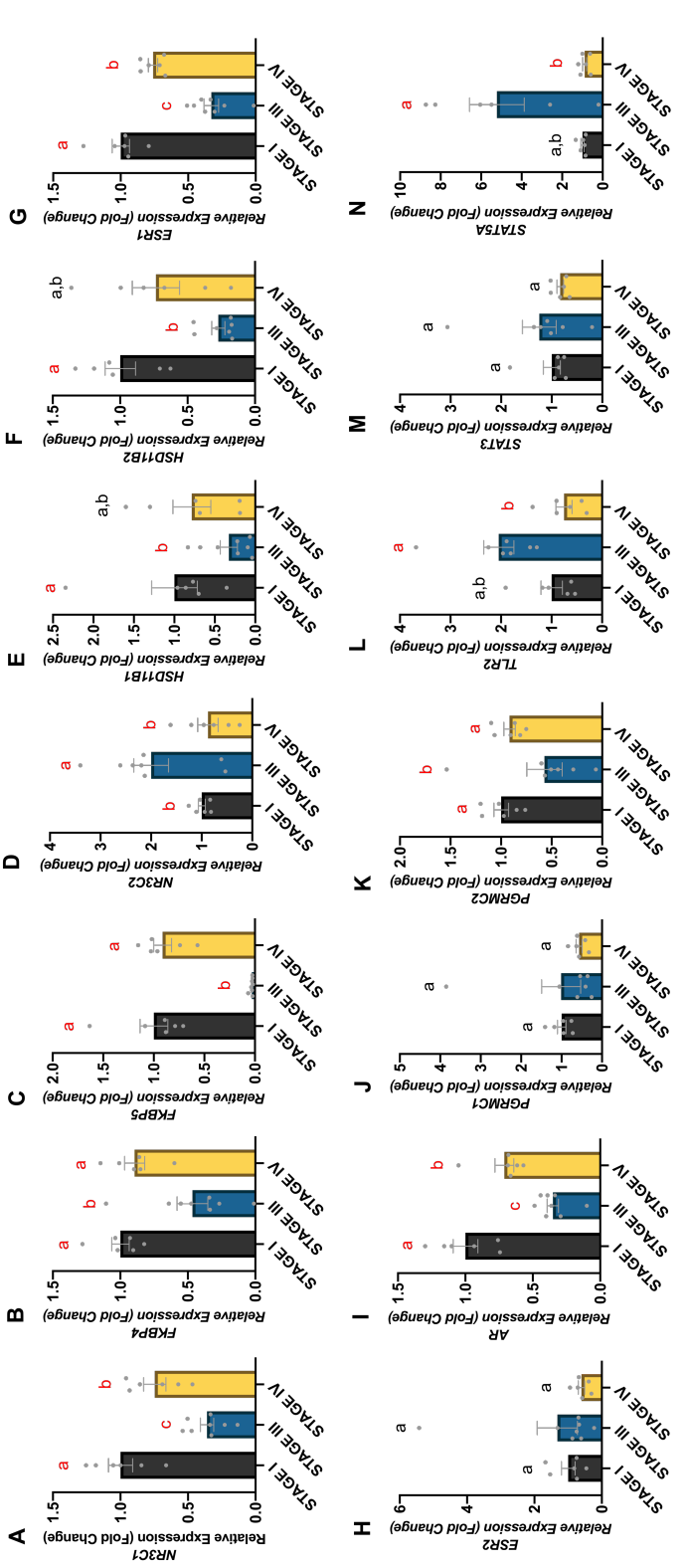

Figure 1. Relative gene expression (fold change) of the target genes (**A**: *NR3C1*; **B**: *FKBP4*; **C**: *FKBP5*; **D**: *NR3C2*; **E**: *HSD11B1*; **F**: *HSD11B2*; **G**: *ESR1*; **H**: *ESR2*; **I**: *AR*; **J**: *PGRMC1*; **K**: *PGRMC2*; **L**: *TLR2*; **M**: *STAT3*; **N**: *STAT5A*) in the ampulla of each individual, comparing stage I (black) vs. stage III (blue) vs. stage IV (yellow); (mean ± SEM). Stage I was set as the reference group. Significant differences are represented by different letters (red; $p < 0.05$).

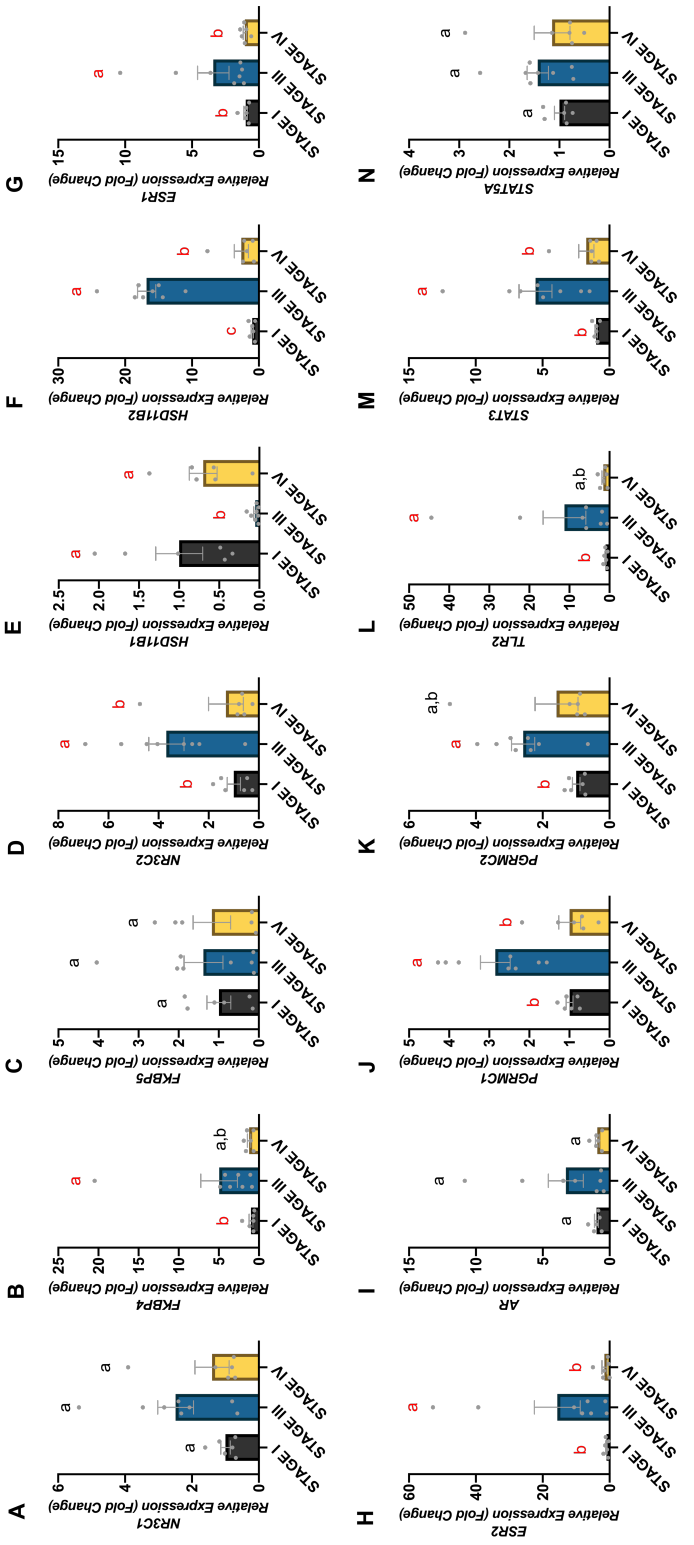

Figure 2. Relative gene expression (fold change) of the target genes (**A**: *NR3C1*; **B**: *FKBP4*; **C**: *FKBP5*; **D**: *NR3C2*; **E**: *HSD11B1*; **F**: *HSD11B2*; **G**: *ESR1*; **H**: *ESR2*; **I**: *AR*; **J**: *PGRMC1*; **K**: *PGRMC2*; **L**: *TLR2*; **M**: *STAT3*; **N**: *STAT5A*) in the endometrium of each individual, comparing stage I (black) vs. stage III (blue) vs. stage IV (yellow); (mean ± SEM). Stage I was set as the reference group. Significant differences are represented by different letters (red; $p < 0.05$).

AMPULLARY AND ENDOMETRIAL mRNA EXPRESSION OF HSD11B1 vs. HSD11B2 DURING EACH ESTROUS CYCLE STAGE

In the ampulla (**Figure 3**; **A**-**C**), higher mRNA expression ($p < 0.05$) of *HSD11B2* was found during stage I, stage III and stage IV compared to *HSD11B1*. In the endometrium (**Figure 3**; **D**-**F**), *HSD11B2* mRNA expression was higher in stage III, but lower in stage I and IV, when compared to *HSD11B1* mRNA expression ($p < 0.05$).

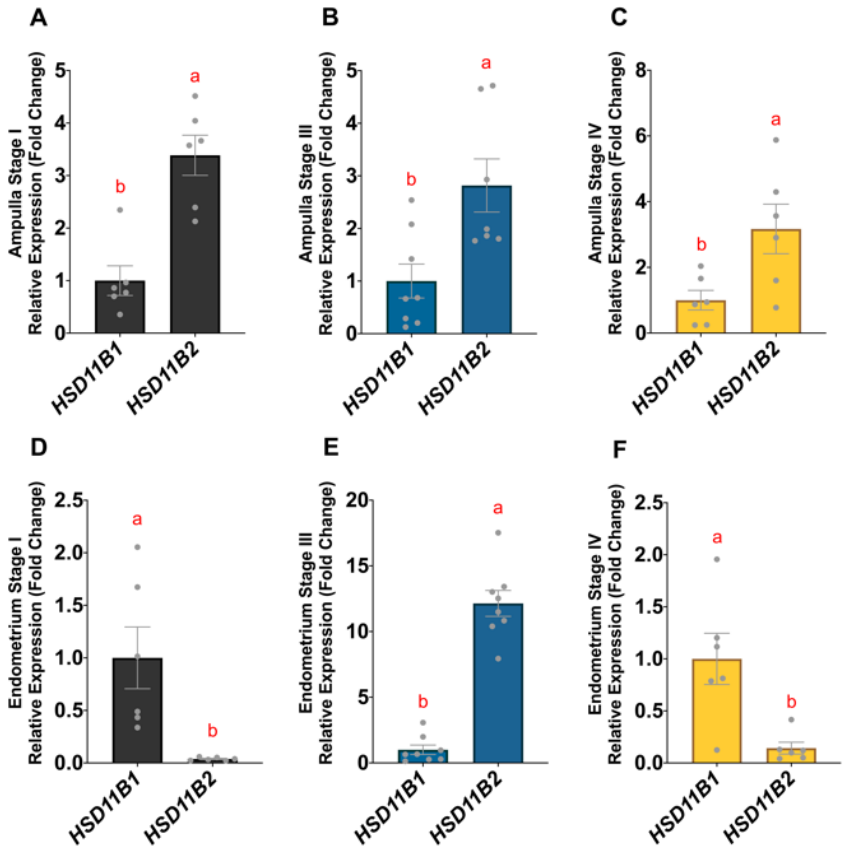

Figure 3. Relative gene expression (fold change) of *HSD11B1* (green) and *HSD11B2* (red), in the ampulla (**A**, **B**, **C**) and endometrium (**D**, **E**, **F**) during stage I (**A**, **D**), stage III (**B**, **E**) and stage IV (**C**, **F**) of the bovine estrous cycle; (mean ± SEM). *HSD11B1* mRNA expression was used as a reference for relative *HSD11B2* expression. Significant differences ($p < 0.05$) are represented by different letters (red).

AMPULLARY AND ENDOMETRIAL mRNA EXPRESSION OF FKBP4 vs. FKBP5 DURING EACH ESTROUS CYCLE STAGE

In the ampulla, higher mRNA expression of *FKPB5* ($p < 0.05$) compared to *FKBP4* (**Figure 4**; **A**-**C**) was observed during stage I and stage IV. On the other hand, in stage III we detected lower mRNA expression levels of *FKPB5* ($p < 0.05$) compared to *FKBP4*. In the endometrium (**Figure 4**; **D**-**F**), lower *FKBP5* during stage III was found when compared to *FKBP4* ($p < 0.05$), while no significative changes were detected during stage IV and stage I.

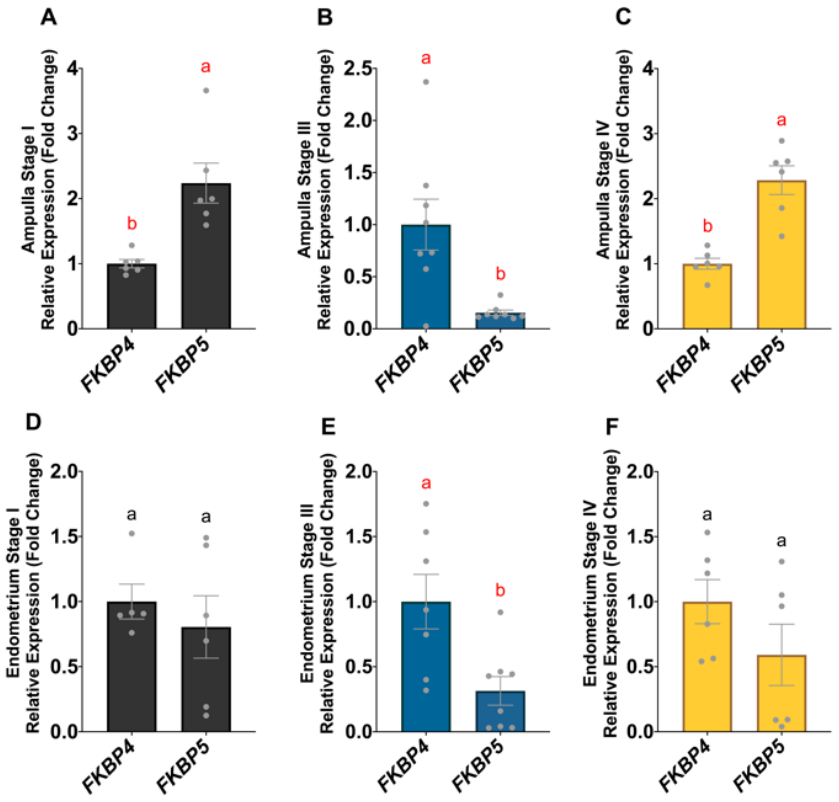

Figure 4. Relative gene expression (fold change) of *FKBP4* (blue) and *FKBP5* (purple), in the ampulla (**A**, **B**, **C**) and endometrium (**D**, **E**, **F**) during stage I (**A**, **D**), stage III (**B**, **E**) and stage IV (**C**, **F**) of the bovine estrous cycle; (mean ± SEM). *FKBP4* mRNA expression was used as a reference for relative *FKBP5* expression. Significant differences ($p < 0.05$) are represented by different letters (red).

CORRELATIONS BETWEEN mRNA EXPRESSION AND P4 AND E2 CONCENTRATIONS

The P4 concentration measured in follicular fluid was 119.69 ± 42.81 ng/mL in stage I; 153.11 ± 95.20 ng/mL in stage III; and 194.49 ± 78.50 ng/mL in stage IV (mean ± SD). Regarding E2, concentrations of 42.15 ± 32.86 ng/mL were observed for stage I; 56.59 ± 72.44 ng/mL for stage III, and 110.32 ± 90.65 ng/mL for stage IV (mean ± SD). Additionally, progesterone/estradiol (P4/E2) ratios were calculated at stage I (6.92 ± 5.94), stage III (11.46 ± 14.1), and stage IV (6.30 ± 10.1).

One-to-one correlations with each mRNA and follicular fluid hormonal concentrations were performed in the ampulla (**Figure 5**; **A-C**) and in the endometrium (**Figure 5**; **D-F**). The highest number of positive and negative significant correlations were found in stage I. During this stage, in the ampulla, *NR3C1* and a high number of genes, including *ESR1*, *AR*, *PGRMC1*, *PGRMC2*, *STAT3*, *STAT5A*, were positively correlated with the P4/E2 ratio, while negatively correlated with E2, *NR3C2*, *ESR2* and *HSD11B1*. In stage III, *ESR2* was correlated with *NR3C1*, in contrast to *ESR1*, that was negatively correlated to *NR3C1* mRNA. Also, *HSD11B1* and *HSD11B2* were positively correlated with *NR3C1* in both stage III and stage IV. In the endometrium, during stage I, a positive correlation was found between *NR3C1* and *HSD11B1*, *HSD11B2*, *PGRMC2*, *NR3C2*, *AR*, *PGRMC1*, *PGRMC2*, while the *NR3C1* mRNA negatively correlated with *FKBP4*, *FKBP5*, *STAT3* and *ESR2*. Interestingly, both *FKBP4* and *FKBP5* were positively correlated with *NR3C1* in the ampulla during stage I, and in the endometrium, both were also positively correlated during stage III. Moreover, a negative correlation was found between *ESR1* and *ESR2* in the ampulla, irrespectively to the estrous cycle phase. In the endometrium, a positive correlation was found between *PGRMC1* and *PGRMC2*, also in all the stages. Finally, E2 was negatively

correlated to *STAT5A* in stage I and stage IV in both tissues, whereas this correlation was positive for stage III. P4/E2 ratio and *PGRMC1* were negatively correlated in both tissues during stage III, but positively correlated during stage IV.

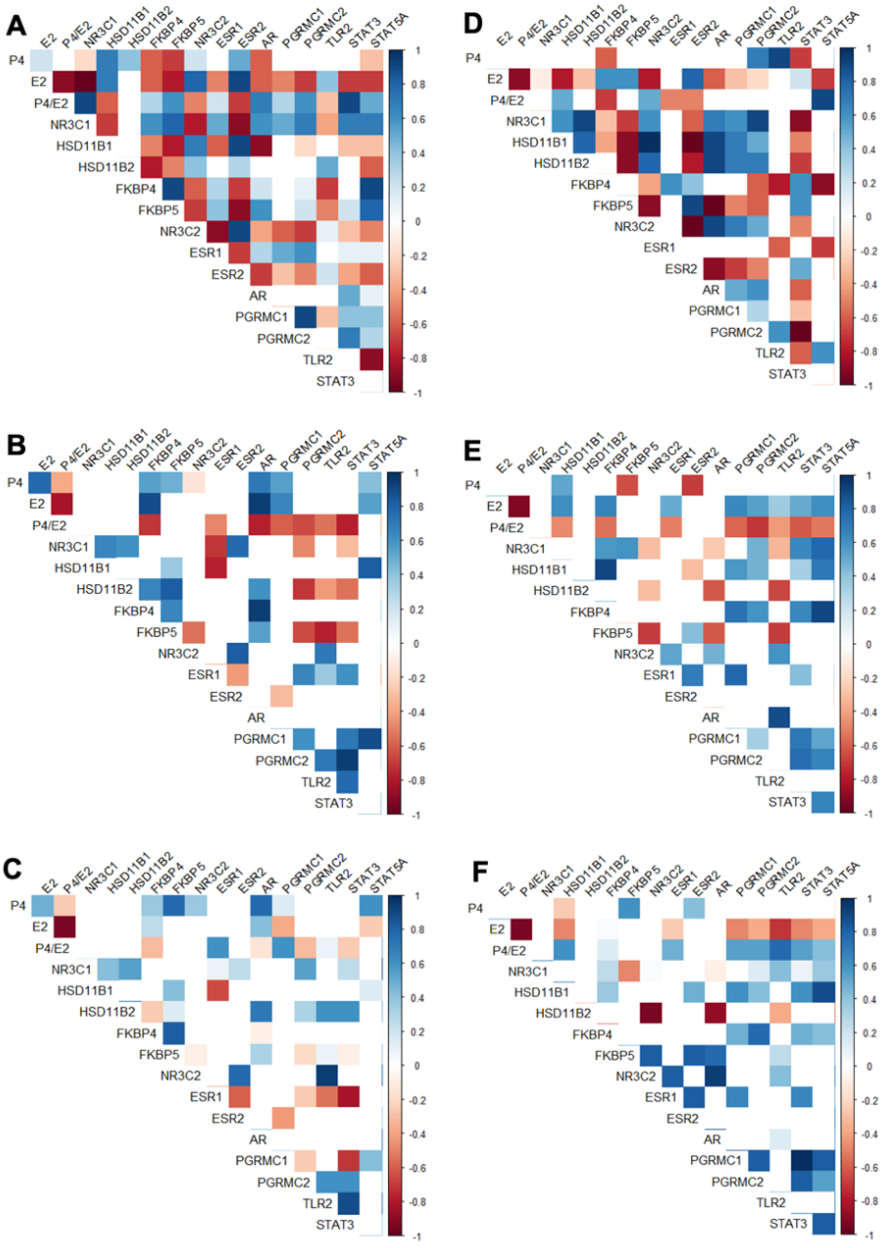

Figure 5. mRNA relative expression one-to-one correlations (Spearman rank) with each transcript, and with follicular fluid estradiol (E2) and progesterone (P4) concentrations, in the ampulla (**A**-**C**) and the endometrium (**D**-**F**). Different stages of the estrous cycle are represented as follows: stage I (**A**, **D**), stage III (**B**, **E**), and stage IV (**C**, **F**). Significant positive correlations are shown as blue squares for each pair of genes or hormones ($p < 0.05$), and significant negative correlation coefficients are shown as red squares for each pair of genes or hormones ($p < 0.05$). Non-significant correlations are shown as empty squares.

AMPULLARY AND ENDOMETRIAL PROTEIN EXPRESSION OF NR3C1 DURING EACH ESTROUS CYCLE STAGE

In the ampulla, we did not detect significant differences ($p < 0.05$) in the NR3C1 protein expression between stages (**Figure 6A**). In the endometrium, we found that NR3C1 levels were significantly higher ($p < 0.05$) in stage IV, compared to stage I and III (**Figure 6B**).

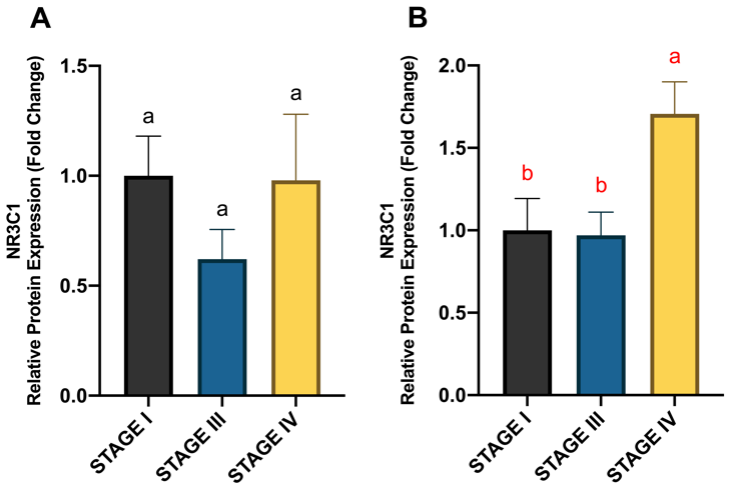

Figure 6. Relative protein expression (fold change) of NR3C1 in the ampulla (**A**) and endometrium (**B**) comparing stage I (black) vs. stage III (blue) vs. stage IV (yellow); (mean ± SEM). Stage I was set as the reference group. Significant differences are represented by different letters (red; $p < 0.05$).

CORRELATION BETWEEN NR3C1 PROTEIN AND mRNA EXPRESSION LEVELS

The NR3C1 protein expression quantified in the ampulla showed significant positive correlation with the mRNA relative expression (Spearman's rank correlation coefficient = 0.51; $p = 0.039$). In the endometrium, correlation between the protein expression levels detected and mRNA levels was not significant (Spearman's rank correlation coefficient = -0.16; $p = 0.528$) (**Figure 7**; **A**-**B**).

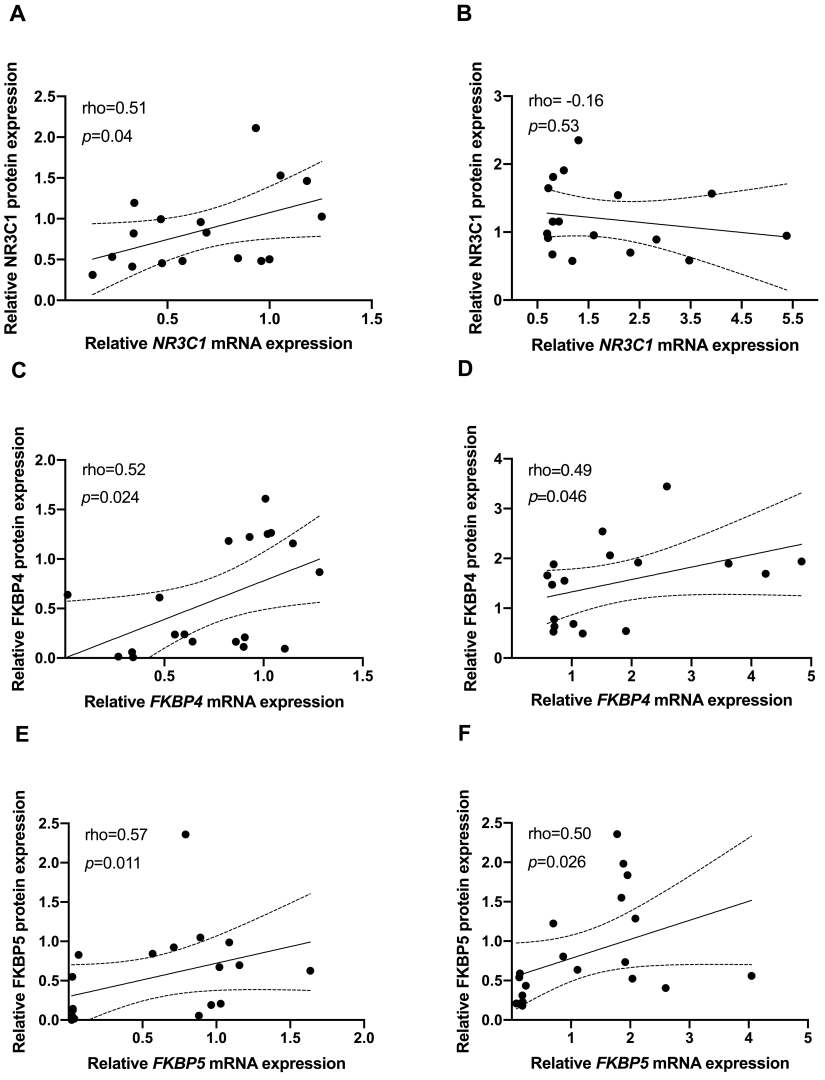

Figure 7. Correlation (Spearman's rank) between protein relative expression and mRNA relative expression of NR3C1 (**A**,**B**), FKBP4 (**C**,**D**) and FKBP5 (**E**,**F**) in each sample of ampulla (**A**,**C**,**E**) and endometrium (**B**,**D**,**F**) in stage I, III and IV. Spearman's rank correlation coefficient (rho) shows the strength of the correlations. Scatter plot and linear regression (line) with 95% confidence interval (dashed line) is represented in each plot and shows the direction of the correlations. The *p* value represents the significance of the correlations ($p < 0.05$).

AMPULLARY AND ENDOMETRIAL PROTEIN EXPRESSION OF FKBP4 AND FKBP5 DURING EACH ESTROUS CYCLE STAGE

The ampullary protein expression levels of FKBP4 (FKBP52) observed in the ampulla were significantly higher ($p < 0.05$) in stage I, compared to stage III (**Figure 8**; **A-B**). Regarding endometrium, we found that the protein expression of FKBP4 was significantly lower ($p < 0.05$) in stage I than in stage III (**Figure 8**; **C-D**). For FKBP5 (FKBP51), the ampullary protein expression levels observed in stage III were significantly lower ($p < 0.05$) in comparison to stage I (**Figure 9**; **A-B**). In the endometrium, we did not find significant differences ($p < 0.05$) in the FKBP5 protein expression between stages (**Figure 9**; **C-D**).

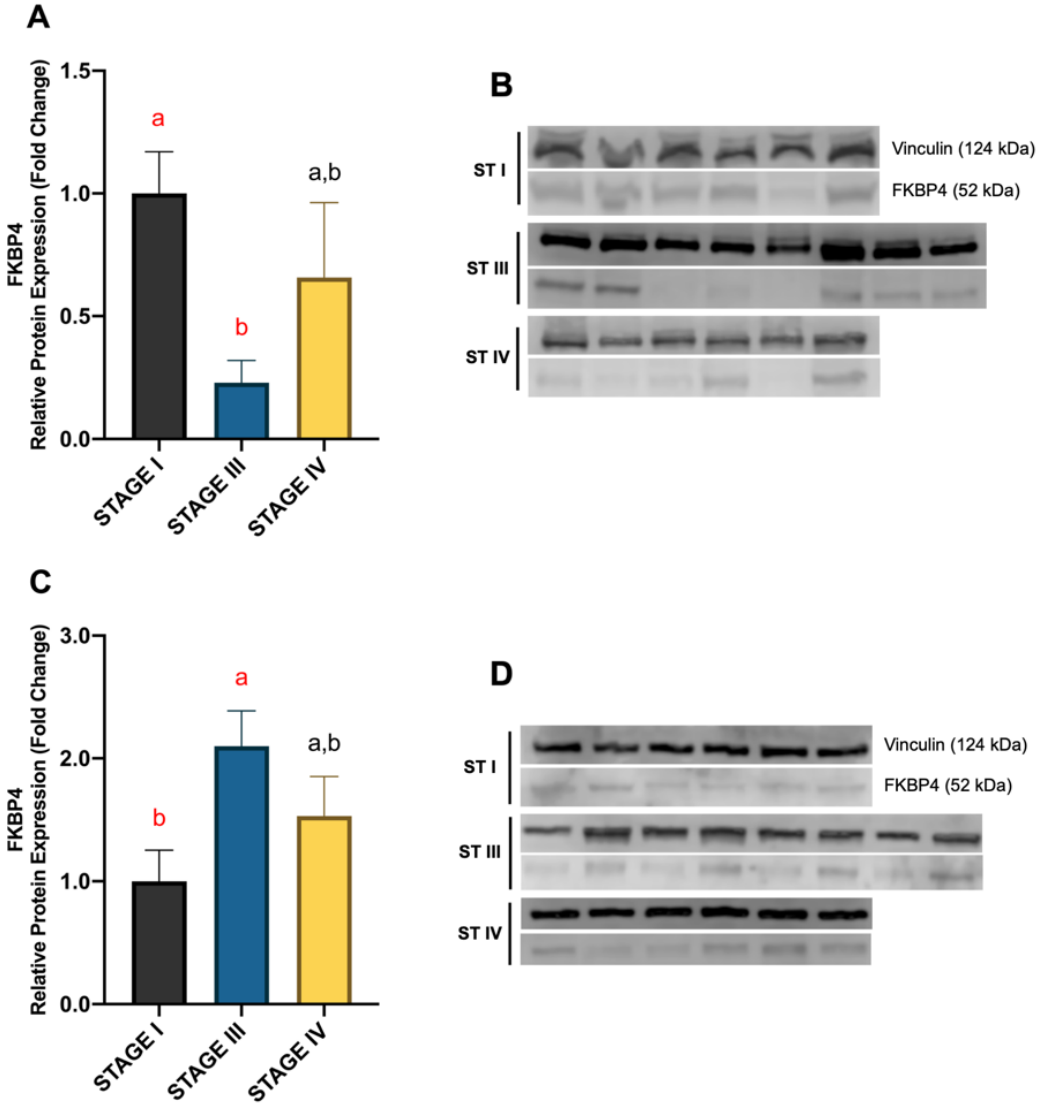

Figure 8. Relative protein expression (fold change) of FKBP4 in the ampulla (**A**) and endometrium (**C**) comparing stage I (black) vs. stage III (blue) vs. stage IV (yellow); (mean ± SEM). Stage I was set as the reference group. Significant differences are represented by different letters (red; $p < 0.01$). Western blot analyses of stage I (ST I), stage III (ST III) and stage IV (ST IV) samples were used for FKBP4 protein (52 kDa) detection and quantification in the ampulla (**B**) and endometrium (**D**). Vinculin (124 kDa) housekeeping protein was used as loading control for Western blot normalization.

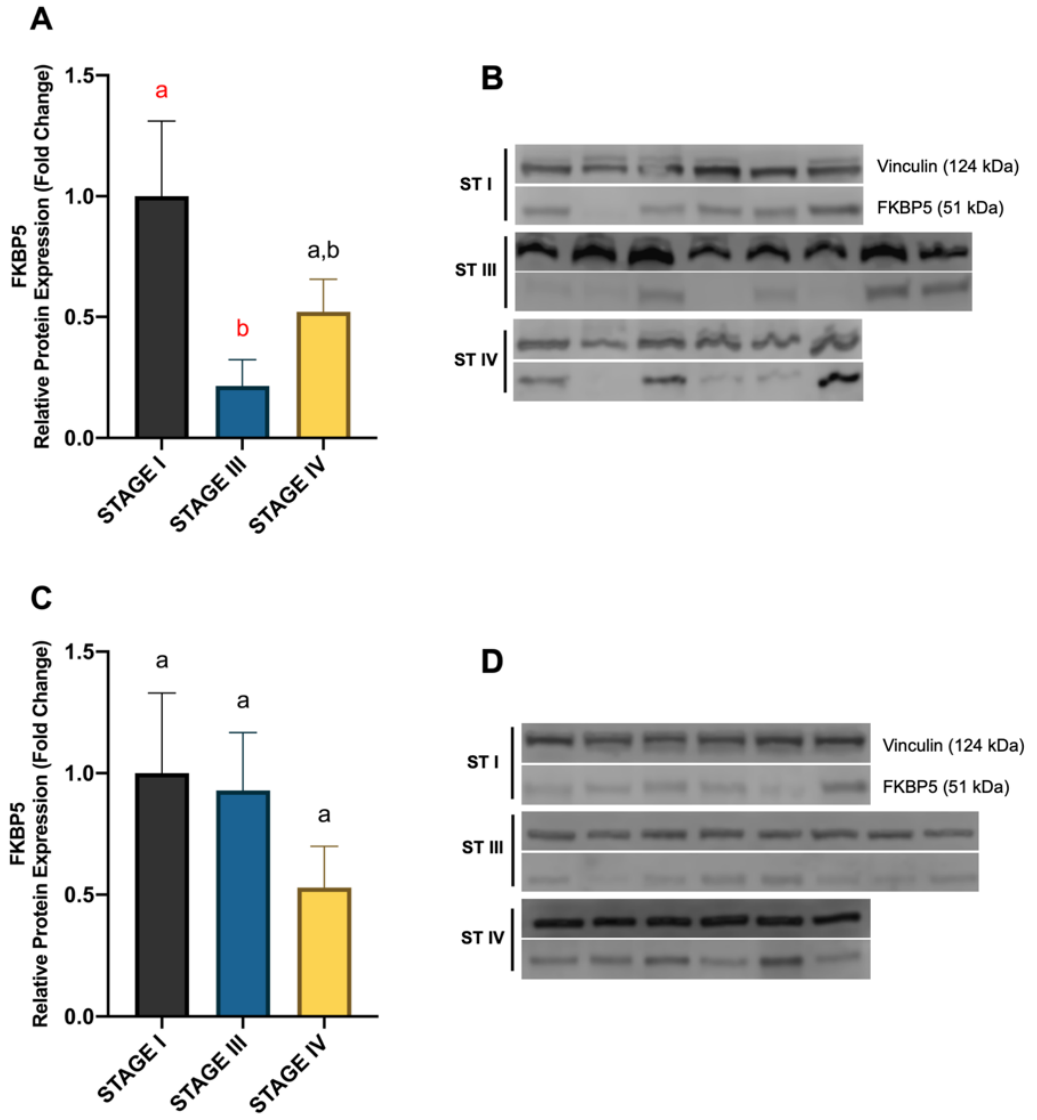

Figure 9. Relative protein expression (fold change) of FKBP5 in the ampulla (**A**) and endometrium (**C**) comparing stage I (black) vs. stage III (blue) vs. stage IV (yellow); (mean ± SEM). Stage I was set as the reference group. Significant differences are represented by different letters (red; $p < 0.05$). Western blot analyses of stage I (ST I), stage III (ST III) and stage IV (ST IV) samples were used for FKBP5 protein (51 kDa) detection and quantification in the ampulla (**B**) and endometrium (**D**). Vinculin (124 kDa) housekeeping protein was used as loading control for Western blot normalization.

CORRELATIONS BETWEEN FKBP4 AND FKBP5 PROTEINS AND THEIR mRNA EXPRESSION LEVELS

The relative FKBP4 protein expression detected in both the ampulla and the endometrium, showed significant positive correlation with their respective ampullary (Spearman's rank correlation coefficient = 0.52; p = 0.024) and endometrial (Spearman's rank correlation coefficient = 0.49; p = 0.046) mRNA relative expression levels (**Figure 7**; **C-D**). Regarding the relative protein expression of FKBP5 levels, we also found a significant positive correlation with their respective mRNA relative expression levels both in the ampulla (Spearman's rank correlation coefficient = 0.57; p = 0.011) and the endometrium (Spearman's rank correlation coefficient = 0.50; p = 0.026) (**Figure 7**; **E-F**).

DISCUSSION

Glucocorticoids (GCs), and also P4 and estrogen levels, modulate the female reproductive tract during the estrous cycle (Whirledge & Cidlowski, 2017). Our results confirm the presence of changes on the GC-related expression during the post-ovulatory phase (stage I) in the ampulla, including an increase in the mRNA expression of *NR3C1*, the main mediator of the GCs actions, and other related genes involved in the GC actions (*HSD11B1*, *HSD11B2, FKBP4* and *FKBP5).* In contrast, endometrial mRNA expression changes were predominantly observed during the late luteal phase (stage III), including lower expression of *HSD11B1* and higher expression in *HSD11B2* and *NR3C2*.

We found that, at the mRNA level, the glucocorticoid receptor *NR3C1* expression is progressively increasing in the ampulla, being higher in the post-ovulatory stage. In this sense, we detected that there is a promotion of *NR3C1*

mRNA expression in the bovine ampulla after ovulation, coinciding in time with the oocyte presence in the oviduct (Siemieniuch *et al.*, 2010). This increase on *NR3C1* during the postovulatory phase can be linked to the local GC environment surrounding the bovine oocyte that is promoted during the oocyte maturation and transport through the oviduct in this species (Tetsuka & Tanakadate, 2019). In this sense, the GCs may have an influence on the oocytes that can be considerably despair depending on the species (Gong *et al.*, 2017; Scarlet *et al.*, 2017; Yang *et al.*, 1999), but seems to be positive for the bovine oocytes (da Costa *et al.*, 2016) and embryos (Santana *et al.*, 2014). Moreover, during the late luteal phase, when the oocyte is no longer in the oviduct and high levels of P4 are present in the reproductive tract (Stevenson & Lamb, 2016), we observed a decrease in the ampullary *NR3C1* mRNA expression, which can be related to the avoidance of the deleterious effects produced by a prolonged exposure to GC (Okret *et al.*, 1986; Rosewicz *et al.*, 1988), as cortisol can be produced from P4 by the action of steroidogenic enzymes (Amweg *et al.*, 2017). In contrast to our results in mRNA, we did not detect differences in the NR3C1 protein expression across the different stages of the estrous cycle in the ampulla. In the endometrium, we did not detect mRNA differences between stages, but an increased expression was found at protein level during the pre-ovulatory phase (stage IV). The presence of NR3C1 in the endometrium, and its regulation during pregnancy has been described in bovine and ovine (Kuse *et al.*, 2013; Simmons *et al.*, 2010), and cortisol production in this tissue has been suggested during this stage (Simmons *et al.*, 2010). Discrepancies in mRNA and protein results are common and can be explained by post-translational modifications that may not be detected by some proteomic approaches. In this sense, variations in the initiation site of translation, rates of protein degradation, and modifications, including ubiquitination, phosphorylation, glycosylation and/or SUMOylation can

produce a vast number of NR3C1 protein isoforms (Druker *et al.*, 2013; Duma *et al.*, 2006; Oakley & Cidlowski, 2011; Tian *et al.*, 2002). While ampullary NR3C1 mRNA and protein abundances were acceptably correlated (51% of the variation in protein abundances can be explained by knowing mRNA levels) (Vogel & Marcotte, 2012), we did not find correlation in the endometrium, meaning that some interferences may be present. Moreover, the presence of particular non detected isoforms that may be playing particular roles in GC function in reproduction should not be discarded (Čikoš *et al.*, 2019).

The availability of GCs binding to NR3C1 is controlled by the HSD11B1 and HSD11B2 enzymes (Chapman *et al.*, 2013; Michael *et al.*, 2003). The *HSD11B1* gene encodes for a bidirectional reductase that converts cortisone metabolites into active cortisol, and the *HSD11B2* does it for an oxidase that catalyzes the opposite reaction (Michael *et al.*, 2003). Both enzymes, HSD11B1/2, have been described in the cattle ovary (Amweg *et al.*, 2013; Tetsuka *et al.*, 2010, 2003), but evidences of their expression in the oviduct remained scarce so far (Siemieniuch *et al.*, 2010; Tetsuka & Tanakadate, 2019). We found expression of both *HSD11B1* and *HSD11B2* in the oviduct ampulla at the mRNA level. Moreover, we detected a decrease in their expression during the luteal phase, compared to what occurs at post-ovulation, indicating tight regulation of the GC availability during the bovine oocyte transport in the oviduct. These enzymes are not equally present across mammalian species, and the presence/absence of HSD11B1 or HSD11B2 may trigger an unequal sensitivity to the deleterious effects of GC on the oocytes and embryos during the initial steps of reproduction (Gong *et al.*, 2017; Yang *et al.*, 1999). In humans, the fallopian tube displays only the HSD11B2-mediated cortisol conversion to cortisone, but not HSD11B1 (Muneyyirci-Delale *et al.*, 2005). In contrast, in bovine, the HSD11B1 is expressed in the cumulus cells, while HSD11B2 seems to be restricted to the oocyte (Tetsuka

et al., 2016; Tetsuka & Tanakadate, 2019). In this sense, HSD11B1-mediated promotion of GC creation in the cumulus cells have positive effects for the bovine oocyte maturation and early embryo development (da Costa *et al.*, 2016; Santana *et al.*, 2014; Tetsuka & Tanakadate, 2019). Interestingly, we observed ampullary 3-fold higher *HSD11B2* mRNA levels compared to the *HSD11B1* levels in all the stages, which may be related to the sensitivity of oocytes to GCs (Gong *et al.*, 2017; Scarlet *et al.*, 2017; Yang *et al.*, 1999) raising questions regarding the oviduct contribution to the GC regulation. Moreover, the *HSD11B1* expression is promoted in the endometrium at the pre- and post-ovulatory phases, when compared to the late luteal phase, suggesting a role of cortisol in preventing excessive uterine prostaglandin production during the pre-ovulatory phase (Lee *et al.*, 2007). On the other hand, during the late luteal phase, cortisol inactivation may be driven by the *HSD11B2* higher expression and *HSD11B1* lower expression that we detected in the endometrium at this stage.

Furthermore, an increased expression of the mineralocorticoid receptor (*NR3C2*) was observed in the endometrium at the late luteal phase. The NR3C2 is a promiscuous receptor that has a higher affinity for cortisol than for other hormones, such as aldosterone (Baker *et al.*, 2013). GCs limitation by the HSD11B2 may allow mineralocorticoids to bind NR3C2 (Chapman *et al.*, 2013). Thus, the NR3C2 signaling is directly implied in the sensitivity of NR3C1 to GCs both by ligand competition, and by modulation of other factors involved in NR3C1, such as the FKBP immunophilins (Hartmann *et al.*, 2021). The NR3C1 regulation is driven also by the immunophilins FKBP5 and FKBP4 (Ratajczak *et al.*, 2015). The FKBP5, together with other co-factors, is bound to the cytosolic inactive receptor multimeric complex, and it is interchanged by FKBP4 after GC ligands are bound to NR3C1 (Wochnik *et al.*, 2005). Thereafter, FKBP4 recruits the dynein motor protein, supporting the translocation of NR3C1 to the nucleus,

where gene transcription is promoted (Davies *et al.*, 2002). NR3C1-mediated expression of *FKBP5* is then activated, reducing the affinity of the receptor for GCs, therefore controlling the response to GC (Ratajczak *et al.*, 2015; Wochnik *et al.*, 2005). We detected higher mRNA expression in the ampulla of both *FKBP4* and *FKBP5* during the pre- and post-ovulatory phases. Thus, FKBP4-mediated translocation of NR3C1 to the nucleus may occur, inducing the activation of *FKBP5*, which was more expressed than *FKBP4* in the pre- and post-ovulatory phases, indicating increased regulation of the NR3C1 actions when the oocyte is being transported throughout the oviduct, compared to the luteal phase. The increased levels of *FKBP5* detected in the ampulla may help to reduce the sensitivity to the deleterious effects of cortisol, similar to what occurs at systemic level in New-World monkeys compared to Old-World species (Scammell *et al.*, 2001; Westberry *et al.*, 2006). At the protein level, the ampullary FKBP5 and FKBP4 were also found decreased during the late luteal phase, but there were no differences regarding the pre-ovulatory phase. These slight discrepancies may be explained by the rates of protein degradation, post-transcriptional modifications, or issues related to the sensitivity of the protein detection. In addition, we found a correlation between both parameters since 52% and 57% of the variation in FKBP4 and FKBP5 protein abundances, respectively, can be explained by the detected mRNA levels in the ampulla (Vogel & Marcotte, 2012). In the endometrium, *FKBP4* observed expression, but not *FKBP5*, was higher during the late luteal phase, both at mRNA and protein level. These results agree with previous findings in mice regarding the crucial role of FKBP4 expression on uterine receptivity, being knockout mutants completely infertile (Tranguch *et al.*, 2005). In this case, mRNA and protein levels displayed similar differences and their levels were also correlated for both FKBP4 and FKBP5 (49% and 50% of the

variation in protein abundances explained by mRNA, respectively) in the endometrium (Vogel & Marcotte, 2012).

The STATs, relevant in the Janus kinase (JAK)/STAT inflammatory signaling pathways, are important in the GCs signaling since they are directly involved in the transcriptional activity of the NR3C1 complex when translocated to the nucleus (Langlais *et al.*, 2012; Newton *et al.*, 2017). STAT3 is critical for the embryo inner cell mass development (Meng *et al.*, 2015) and the endometrial adherence and trophoblastic invasion (Marconato *et al.*, 2012). It is also activated by potent anti-inflammatory cytokines (Hedl *et al.*, 2019), having a role in the decrease of inflammatory responses (Petta *et al.*, 2016). Our results presented an increase in the endometrium during the late luteal phase, which may correspond to avoidance of potential inflammatory damage. Regarding STAT5A, which is related to inflammatory cytokines, T-cell differentiation, reproduction and pregnancy (Bednorz *et al.*, 2011; Maj & Chelmonska-Soyta, 2007), we observed a decreased expression during the pre-ovulatory phase, which might contribute to the promotion of tolerance towards spermatozoa in the ampullar region. Moreover, while STAT3 stimulates the transcriptional activity of NR3C1, AR and ESR, STAT5A is involved in NR3C1 recruitment and transcriptional synergism (Langlais *et al.*, 2012; Petta *et al.*, 2016), together with the NF-κB cells, and other factors, enhancing TLR2 expression (Hermoso *et al.*, 2004). We observed an increase in *TLR2* mRNA during the late luteal phase that may contribute to the creation of an adequate uterine environment by polymorphonuclear neutrophil activation, which is needed for protection against pathogens and remaining sperm removal (Alderton, 2012; Ezz *et al.*, 2019). In this context, we detected decreased *TLR2* levels during the pre-ovulatory phase vs. the late luteal phase in the ampulla, which might be associated to lower neutrophil activity prior to ovulation on this region.

Sex steroids, androgen receptor (*AR*) and estrogen receptor 1 (*ESR1*), mRNA expressions in the ampulla were observed increased in the pre-ovulatory phase. Higher levels were detected during the post-ovulatory phase, decreasing during the late luteal phase. This findings differ from previous findings that described stable expression of estrogen and progesterone receptors in the ampulla across the bovine estrous cycle (Ulbrich *et al.*, 2003). The results observed on *ESR1* in the post-ovulatory phase may be linked to the oviductal transport of the oocyte and embryo (Li *et al.*, 2017), and also the sperm migration. In this context, *ESR1* knockout mice reduced by 50% the fertilization rates and produced an impaired oviduct environment for the cumulus cells (Winuthayanon *et al.*, 2015). Regarding the *AR*, it is known to be promoted by both E2 and androgens, and it remains static across menstrual cycles in the fallopian tube in humans (Horne *et al.*, 2009). For progesterone receptor component 2 (*PGRMC2),* we detected a decrease in the ampulla during the late luteal phase, which may be associated to oocyte development (Kowalik *et al.*, 2016; Qiu *et al.*, 2008), and oviductal transport (Nutu *et al.*, 2007). Gene expression studies in the canine oviduct found higher expression in the mRNA levels of both *PGRMC1* and *PGRMC2* during the periovulatory period of the estrous cycle (Tahir *et al.*, 2013), but we only found differences in the *PGRMC2*. In the endometrium, we observed that the mRNA expression of all four estrogen and progesterone receptors included in the present study (*ESR1, ESR2, PGRMC1, PGRMC2*) increased at the late luteal phase. Relevant activity for correct physiology may be exerted by steroid receptors during the late luteal phase, when P4 levels are elevated, and E2 levels are starting to increase (Forde & Lonergan, 2012). In contrast, *PGRMC1* does not change across the cycle, and reduced levels of *PGRMC2* have been related to endometriosis in macaques (Keator *et al.*, 2012). In addition, P4 levels mediated by progesterone receptors have recently been linked to a beneficial induction of

cortisol production in the bovine oocyte during maturation (Anbo *et al.*, 2022), which may encourage further research regarding the interplay between the actions of steroid receptors in reproduction.

CONCLUSIONS

In conclusion, there is an important modulation of the GC regulatory activity in the bovine reproductive tract during the estrous cycle both in the ampulla and the endometrium. GC activity is promoted in the ampulla during the post-ovulatory phase (stage I) by increasing mRNA expression of *NR3C1*, *FKBP4* and *FKBP5*, *HSD11B1* and *HSD11B2*, claiming for a role of the oviduct in the regulation of GC actions during early reproductive events taken place on this region, including oocyte maturation. In the endometrium important changes in the GC-related mRNA expression occurred during the late luteal phase (stage III), by the time that the embryonic maternal recognition is established in the uterus. Further studies should focus on mechanistic studies to elucidate the specific contribution of each of the agents involved in the crucial steroid regulation taking place in the female reproductive tract.

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Declaration of interest: None.

Author contributions: MRC: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing – original Draft, Visualization; JG: Methodology, Investigation, Writing – Review & Editing; SOM: Methodology, Investigation, Writing – Review & Editing; MLB: Conceptualization,

Resources, Writing – Review & Editing, Supervision, Funding acquisition; MAR: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Supplementary Table 1. Values of RNA purity (RNA 260/230 and 260/280 absorbance ratios) and RNA integrity (RIN values).

Tissue	Sample ID	Stage	RNA 260/230	RNA 260/280	RIN Values
Ampulla	1A	I	1.89	2.05	9.2
Ampulla	2A	I	1.98	2.03	8.9
Ampulla	3A	I	2.16	2.04	8.9
Ampulla	4A	I	1.73	2.04	9.3
Ampulla	5A	I	2.17	2.07	8.9
Ampulla	6A	I	1.76	2.01	8.8
Ampulla	7A	III	1.86	2.00	9.1
Ampulla	8A	III	1.75	1.97	8.9
Ampulla	9A	III	1.76	2.04	8.2
Ampulla	10A	III	2.14	1.98	8.6
Ampulla	11A	III	1.95	1.98	8.7
Ampulla	12A	III	2.02	1.97	8.7
Ampulla	13A	III	1.72	1.98	8.9
Ampulla	14A	III	1.91	1.98	8.4
Ampulla	15A	IV	1.83	2.01	9.3
Ampulla	16A	IV	1.80	2.00	9.3
Ampulla	17A	IV	2.12	2.06	8.9
Ampulla	18A	IV	1.73	2.07	9.4
Ampulla	19A	IV	1.85	1.84	8.8
Ampulla	20A	IV	2.10	2.04	9.5
Endometrium	1E	I	1.86	2.00	8.1
Endometrium	2E	I	1.82	2.00	8.1
Endometrium	3E	I	2.02	2.03	8.8
Endometrium	4E	I	2.03	2.13	8.2
Endometrium	5E	I	1.82	1.98	8.1
Endometrium	6E	I	1.72	1.96	8.6
Endometrium	7E	III	2.12	1.96	8.1
Endometrium	8E	III	1.84	1.95	8.8
Endometrium	9E	III	2.14	2.05	8.5
Endometrium	10E	III	2.21	1.99	8.6
Endometrium	11E	III	1.96	2.00	9.2
Endometrium	12E	III	2.21	1.99	8.8
Endometrium	13E	III	2.01	2.00	9.1
Endometrium	14E	III	1.82	2.04	9.4
Endometrium	15E	IV	2.06	2.01	8.2
Endometrium	16E	IV	1.94	2.00	8.3
Endometrium	17E	IV	2.06	1.95	8.1
Endometrium	18E	IV	1.71	2.00	8.7
Endometrium	19E	IV	1.82	2.04	8.1
Endometrium	20E	IV	1.70	2.00	8.2

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CHAPTER IV

Natural Mating Differentially Triggers Expression of Glucocorticoid Receptor (*NR3C1*)-Related Genes in the Preovulatory Porcine Female Reproductive Tract

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ABSTRACT

Mating initiates dynamic modifications of gene transcription in the female reproductive tract, preparing the female for fertilization and pregnancy. Glucocorticoid signaling is essential for the homeostasis of mammalian physiological functions. This complex glucocorticoid regulation is mediated through the glucocorticoid receptor, also known as nuclear receptor subfamily 3 group C member 1 (NR3C1/GR) and related genes, like 11β-hydroxysteroid dehydrogenases (HSD11Bs) and the FK506-binding immunophilins, FKBP5 and FKBP4. This study tested the transcriptome changes in NR3C1/GR regulation in response to natural mating and/or cervical deposition of the sperm-peak ejaculate fraction collected using the gloved-hand method (semen or only its seminal plasma), in the preovulatory pig reproductive tract (cervix to infundibulum, 24 h after mating/insemination/infusion treatments). Porcine cDNA microarrays revealed 22 *NR3C1*-related transcripts, and changes in gene expression were triggered by all treatments, with natural mating showing the largest differences, including *NR3C1*, *FKBP5*, *FKBP4*, hydroxysteroid 11-beta dehydrogenase 1 and 2 (*HSD11B1*, *HSD11B2*), and the signal transducer and activator of transcription 5A (*STAT5A*). Our data suggest that natural mating induces expression changes that might promote a reduction of the cortisol action in the oviductal sperm reservoir. Together with the STAT-mediated downregulation of cytokine immune actions, this reduction may prevent harmful effects by promoting tolerance towards the spermatozoa stored in the oviduct and perhaps elicit spermatozoa activation and detachment after ovulation.

Keywords: transcriptomics; microarrays; spermatozoa; mating; glucocorticoid; *FKBP5*; *FKBP4*; *NR3C1*; female reproductive tract; pig.

INTRODUCTION

Glucocorticoids (GC) are steroid hormones essential for adaptation to stress, behavior and reproduction. GC release is under circadian/ultradian pulsatile control of the hypothalamic-pituitary-adrenal (HPA) axis and its regulation is absolutely necessary for animal homeostasis. Undeniable new evidence suggests a crucial role of GC during different reproductive steps, where they display central and peripheral regulation (Whirledge & Cidlowski, 2017). The balance between high and low levels of GCs determine whether its biological activity mediates the correct functions or causes pathology. In this sense, it has long been assumed that GCs act negatively on reproductive function due to their role in chronic stress physiology (Tilbrook *et al.*, 2000). However, while high chronic levels of the hormone in response to stress are pathological and affect fertility, basal levels of GC are also essential for normal reproduction (Whirledge & Cidlowski, 2017). For instance, GCs undergo a significant rise prior to ovulation in most mammalian species, and although their increase may lead to impairment of their reproductive function (Whirledge & Cidlowski, 2013), their presence is essential during embryonic/fetal development, parturition and lactation (Fanson & Parrott, 2015; Whirledge & Cidlowski, 2010; Whirledge & Cidlowski, 2017). GCs are also relevant for male fertility, and while GC-exposure drives the inhibition of steroidogenesis in the testis and apoptosis of germ cells (Yazawa *et al.*, 2000), its deficiency impairs testicular function (Kowal *et al.*, 2006; Whirledge & Cidlowski, 2010). Although many studies address their influence in animal reproduction (Lee *et al.*, 2007; Siemieniuch *et al.*, 2010; Simmons *et al.*, 2010; Whirledge *et al.*, 2015), the mechanisms by which GCs participate in energetically-demanding processes (Romero, 2002) (including sperm transport and storage in the female reproductive

tract, the modulation of the immune response, and also their involvement in gene transcription signaling) are still not well known.

Reproductive events that are initiated by either natural mating or artificial insemination (AI), such as sperm transport, sperm storage, fertilization and the cascade of embryonic/fetal processes associated with implantation, placentation, cervical ripening and final delivery, are all accompanied by inflammatory, immunological and transcriptional responses in the reproductive tract. These events are often modulated by GCs, whose regulatory signaling action seem to be mediated by one particular receptor, the glucocorticoid receptor. This receptor, also known as the nuclear receptor subfamily 3 group C member 1 (NR3C1/GR), binds to GC forming a complex that is translocated to the nucleus of the cells to modify gene transcription (Wang & Harris, 2015). This translocation is done with the aid of partner molecules (Ratman *et al.*, 2013; Simmons *et al.*, 2010; Vanderbilt *et al.*, 1987), including the peptidyl-prolyl cis/trans isomerase FK506-binding proteins (FKBP family proteins), which may regulate this translocation (Kang *et al.*, 2008). Thus, cortisol-glucocorticoid receptor complex are responsible for the activation or repression of transcription of target genes (up to 10-20% of the whole genome in humans) (Galon *et al.*, 2002; Oakley & Cidlowski, 2013) triggering, in turn, cascades with pleiotropic implications, that include reproductive, immune and transgenerational effects (Schmidt *et al.*, 2019a; Whirledge *et al.*, 2015).

In this regard, natural mating shapes reproductive physiology in a variety of different species due to a combination of the sensorial stimulation produced by penile buffeting, the presence of spermatozoa and/or the contact with seminal plasma (SP) (Apichela *et al.*, 2014; Orihuela *et al.*, 2001; Shafik *et al.*, 2006). The aforementioned mating factors seem to change gene and protein expression

post-coitus, consequently modulating uterine and oviductal functions (Fazeli *et al.*, 2004). Such modifications elicited in the reproductive tract help to create a suitable environment necessary for sperm storage (Almiñana *et al.*, 2014), gamete transport, pre-implantation development, increased angiogenesis, and also changes in the immune system pattern (Álvarez-Rodríguez *et al.*, 2020; Parada-Bustamante *et al.*, 2016), including the attainment of a status of tolerance to foreign proteins and cells. Although GC signaling is essential for the establishment and maintenance of fertility (Whirledge & Cidlowski, 2017), the up-stream triggers causing the modifications of *NR3C1*-related genes after natural mating or insemination in the preovulatory phase are yet to be determined.

Therefore, the present study tested the hypothesis that (i) natural mating, (ii) the cervical deposition via AI of the sperm-peak ejaculate fraction, and (iii) AI of the sperm-free SP, all equally affect the expression of 22 genes involved in NR3C1/GR regulation in the preovulatory pig reproductive tract, 24 h after treatment. These treatments were performed on samples of mature fertile boars (n = 5) collected using the gloved-hand method.

RESULTS

GENE ONTOLOGY OF THE GENES RELATED TO THE GLUCOCORTICOID RECEPTOR NR3C1

The 22 *NR3C1*-related genes of interest, directly or indirectly engaged in glucocorticoid receptor NR3C1 action and being potentially involved in reproductive functions and signaling (**Figure 1**), were analyzed. Besides *NR3C1*, these genes included the following: hydroxysteroid 11-beta dehydrogenase 1 and 2 (*HSD11B1* and *HSD11B2*), the FK506-binding prolyl isomerase 5 and 4 (*FKBP5*

and *FKBP4*), the prostaglandin-endoperoxide synthase 1 and 2 (*PTGS1* and *PTGS2*), the phospholipase A2 group IV B (*PLA2G4B*), the insulin-like growth factor binding protein 1 (*IGFBP1*), the heat shock protein family A (*HSP70*) member 8 (*HSPA8*), the signal transducer and activator of transcription 1, 2, 3, 5A, 5B, and 6 (*STAT1*, *STAT2*, *STAT3*, *STAT5A*, *STAT5B*, and *STAT6*), the tumor protein P53 (*TP53*), the mediator complex subunit 1 and 14 (*MED1* and *MED14*), the heat shock protein family A (*HSP70*) member 4 and member-4-like (*HSPA4* and *HSPA4L*), and heat shock protein 90 alpha family class B member 1 (*HSP90AB1*).

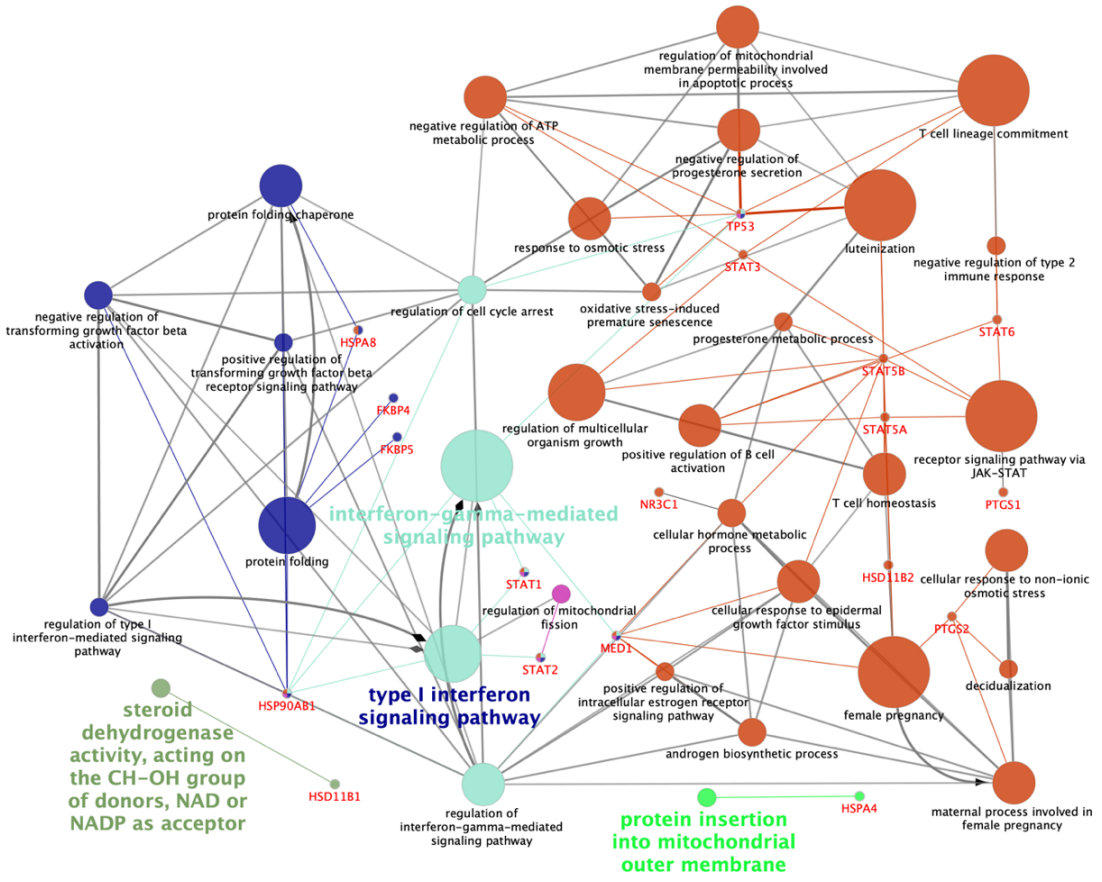

Figure 1. Schematic representation of altered transcripts of interest among all tissues and treatments. The analysis of over-represented functional categories was performed using the Cytoscape v3.0.0 application ClueGo v2.0.3. The following databases were used: Gene Ontology (GO) subgroups biological process which is shown as circles. Terms are functionally grouped based on shared genes (kappa score) and are shown in different colors. The size of the nodes indicates the degree of significance, where the biggest nodes correspond to the highest significance. The most significant term defines the name of the group. The following ClueGo parameters were used: biological process database (BP; date: 19.05.2020); GO tree levels, 2–4 (first level = 0); minimum number of genes, 1; minimum percentage of genes, 1.2; GO term fusion; GO term connection restriction (kappa score), 0.4; GO term grouping, initial group size of 2 and 50% for group merge. The resulting network representation was manually rearranged after removing unnecessary terms. *HSD11B1* and *HSD11B2* (hydroxysteroid 11-beta dehydrogenase 1 and 2), *HSPA4*, *HSPA8* and *HSP90AB1* (heat shock proteins 4, 8 and 90 alpha family class B member 1), *FKBP5* and *FKBP4* (FK506-binding prolyl isomerase 5 and 4), MED1 (mediator complex subunit 1), *NR3C1* (nuclear receptor subfamily 3 group C member 1), *PTGS1* and *PTGS2* (prostaglandin-endoperoxide synthase 1 and 2), *STAT1, STAT2, STAT3, STAT5A, STAT5B* and *STAT6* (signal transducer and activator of transcription 1, 2, 3, 5A, 5B, and 6), *TP53* (tumor protein 53).

NATURAL MATING AND AI OF SEMEN COMPONENTS ALTERED THE EXPRESSION OF GENES RELATED TO THE GLUCOCORTICOID RECEPTOR NR3C1

Figure 2 shows the genes related to the glucocorticoid hormone receptor NR3C1 that were differentially expressed ($p < 0.05$) in the proximal uterus (ProxUt), utero-tubal junction (UTJ), and isthmus (Isth) of the sow reproductive tract 24 h after (i) natural mating, (ii) cervical insemination with the first portion of sperm-rich fraction (semen-AI), or (iii) cervical infusion with the sperm-free seminal plasma of this portion (SP-AI). Interestingly, some of the genes were similarly downregulated (*HSD11B1*, *FKBP4*, *PTGS2*, and *STAT5A*) or upregulated (*FKBP5*) in these three genital compartments, considered the most biologically relevant during the preovulatory phase (Atikuzzaman *et al.*, 2017; Rodriguez-Martinez, 2007). The overall differentially-expressed genes, from cervix (Cvx) to infundibulum (Inf) are represented in **Supplementary Figure S1**.

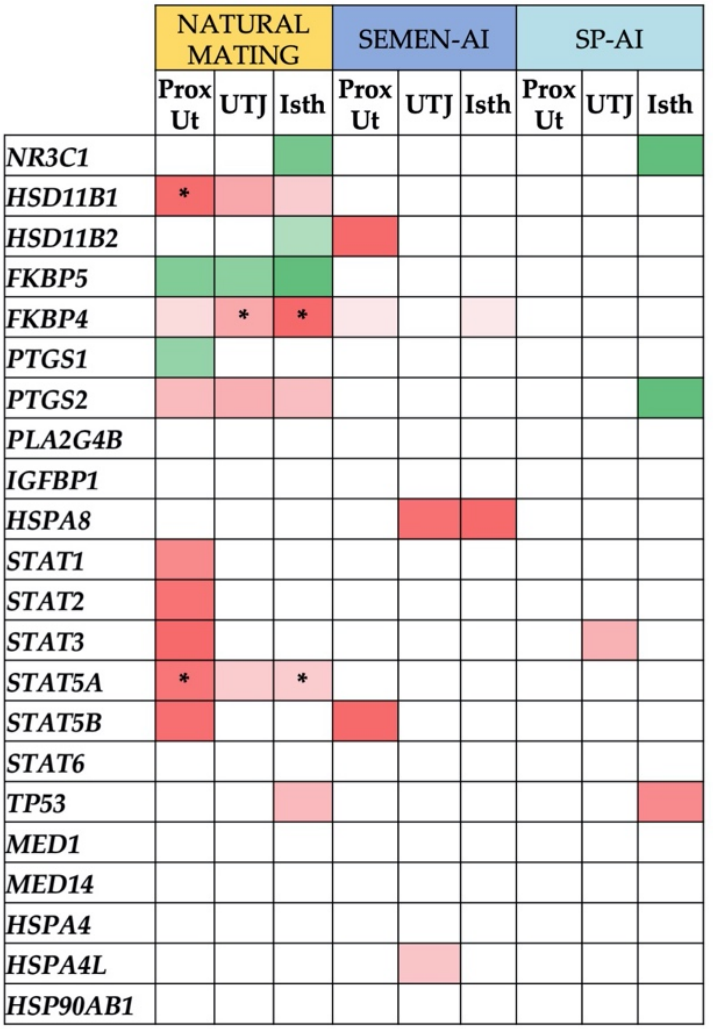

Figure 2. Differentially-expressed genes (DEGs) in proximal uterus (ProxUt), utero-tubal junction (UTJ), and isthmus (Isth) tissues, ordered by treatments (natural mating, semen-artificial insemination (AI), or sperm-free (SP-AI)). Upregulated DEGs are shown in green and downregulated genes are shown in red ($p < 0.05$). Color grading is displayed and a higher up/downregulation of DEGs is represented by more color intensity, while a lower up/downregulation is represented by less color intensity, separately for every treatment. False discovery rates (FDR) ($q < 0.05$) are noted with an asterisk.

Natural mating (**Figure 3**) was, by far, the treatment that caused the largest changes to gene expression. In mating, 23 differential expression changes were registered (10 in ProxUt, five in UTJ, and eight in Isth), with 17 out of the 23

affected genes being downregulated (73.9%) in the reproductive tract segments ($p < 0.05$). Remarkably, all significant genes identified using an FDR (false discovery rate)-corrected threshold ($q < 0.05$) were solely found in the natural mating treatment group (five differential expression changes). In contrast, in the semen-AI group only seven differential expression changes were found ($p < 0.05$), all of them being downregulation. Regarding the seminal plasma treatment (SP-AI), there were a total of four differential expression changes ($p < 0.05$), two of them being downregulated (50%).

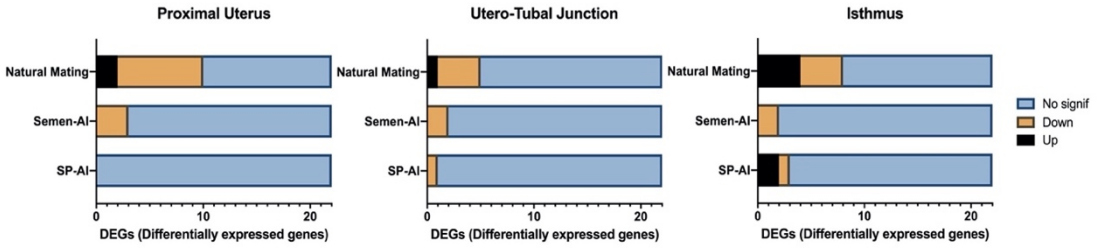

Figure 3. Number of differentially-expressed genes (DEGs) present in proximal uterus, utero-tubal junction, and isthmus tissues. Upregulated genes ($p < 0.05$) are represented in black and downregulated genes ($p < 0.05$) in orange color. Non-significant genes are represented in blue.

Thus, in ProxUt tissue (**Figure 2**), natural mating upregulated *FKBP5* and *PTGS1* but downregulated *FKBP4*, *PTGS2*, *HSD11B1* ($q < 0.05$), *STAT1*, *STAT2*, *STAT3*, *STAT5A* ($q < 0.05$), and *STAT5B* ($p < 0.05$). Semen-AI downregulated *HSD11B2*, *FKBP4*, and *STAT5B* ($p < 0.05$) while SP-AI infusion resulted in no differentially-expressed genes (DEGs).

In the UTJ (**Figure 2**), considered the main functional oviductal sperm reservoir in pigs, natural mating also upregulated the *FKBP5* gene ($p < 0.05$) while downregulating *FKBP4* ($q < 0.05$), *HSD11B1*, *PTGS2*, and *STAT5A* ($p < 0.05$). The

other treatments using AI downregulated ($p < 0.05$) *HSPA8* and *HSPA4L* in semen-AI, and *STAT3* in the case of SP-AI.

In the Isth tissue (**Figure 2**), natural mating upregulated *FKBP5*, *NR3C1*, and *HSD11B2* ($p < 0.05$), while downregulating *FKBP4* ($q < 0.05$), *HSD11B1*, *PTGS2*, *STAT5A* ($q < 0.05$), and *TP53* ($p < 0.05$). The semen-AI treatment downregulated *FKBP4* and *HSPA8* ($p < 0.05$) while the SP-AI infusion treatment upregulated *NR3C1* and *PTGS2* ($p < 0.05$) and downregulated *TP53* ($p < 0.05$).

Regarding the rest of the tissues tested, in the Cvx, only sperm-containing treatments induced a change in gene expression, downregulating all genes ($p < 0.05$). Natural mating induced downregulation of *FKBP4*, *HSD11B1*, *PTGS2*, *STAT1*, *STAT2*, *STAT5A*, *TP53*, and *HSPA4* ($p < 0.05$) while semen-AI downregulated *HSPA4L* ($p < 0.05$). In the distal uterus (DistUt), natural mating induced a similar expression pattern to that seen in the ProxUt tissue (7/3 and 8/2 down/upregulated genes, respectively). Here, *FKBP5*, *HSP4L* ($q < 0.05$), and *HSP90AB1* were upregulated while *FKBP4*, *HSD11B1* ($q < 0.05$), *STAT1*, *STAT2*, *STAT3*, *STAT5A* ($q < 0.05$), and *STAT6* ($p < 0.05$) were downregulated. Interestingly, both semen-AI (first portion of sperm-rich fraction) and its sperm-free seminal plasma (SP-AI) treatments, induced *PTGS1*-downregulation in the DistUt ($p < 0.05$). The seminal plasma treatment (SP-AI) induced upregulation of *HSPA*4L ($p < 0.05$). The ampulla (Amp) and Inf were the tissues showing most DEGs. Natural mating displayed a similar expression pattern in the two tissues with *FKBP4* ($q < 0.05$), *HSPA8*, *HSPA4L*, *HSP90AB1*, and *MED14* being downregulated ($p < 0.05$) and *HSD11B2* ($q < 0.05$ in Inf), *STAT3* ($q < 0.05$ in Amp), and *STAT6* ($q < 0.05$ in Inf), being upregulated ($p < 0.05$). The *PLA2G4B* and *PTGS1* genes were upregulated in Amp in natural mating ($p < 0.05$).

Additionally, in the Amp, *PLA2G4B* was also upregulated by semen-AI and SP-AI treatments ($p < 0.05$). Ampullar downregulation of *MED1* was induced by natural mating and semen-AI treatments ($p < 0.05$). Semen-AI induced downregulation of *HSPA8, HSPA4*, and *STAT1* and upregulated *STAT6* ($p < 0.05$) while the SP-AI treatment only downregulated *STAT1* ($p < 0.05$). In the Inf, the sperm-containing treatments (natural mating and semen-AI) upregulated *NR3C1* ($p < 0.05$) while the semen-AI treatment also upregulated *PLA2G4B* ($p < 0.05$). Similar to the other tissues, *STAT5A* was downregulated by natural mating ($p < 0.05$) while *HSPA8*, *FKPB4*, and *HSPA4* were downregulated by semen-AI and natural mating ($p < 0.05$). *STAT5A* and *TP53* were downregulated by SP-AI ($p < 0.05$).

Additionally, the principal component analysis (PCA, (Jollife, 2014)), showed that in the ProxUt, the first principal component (PC1) explained 34.6% and the second principal component (PC2) 24% of the total variance ($n = 16$). For the UTJ, PC1 and PC2 explained 25.7% and 22.2%, respectively ($n = 16$). In the case of Isth, PC1 explained 28% and PC2 explained 18% ($n = 16$) (see **Supplementary Figure S2**). A heat map representation for the ProxUt, UTJ, and Isth tissues was performed to aid visualization of group separation. Data was clustered using correlation distance and average linkage (see **Supplementary Figure S3**).

KEGG PATHWAY ANALYSIS

Differentially-expressed genes were annotated into different biological pathways by using the official names of the genes and the *Sus scrofa* annotation in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database (Kanehisa *et al.*, 2019). The KEGG pathways enabled the analysis and organization of the detected

genes according to their signaling pathways. The data was organized by treatment, and by the total number of genes analyzed (22), 21 genes (95%) were represented in the natural mating group (**Supplementary Table S1**), 12 genes (55%) in the semen-AI group (**Supplementary Table S2**), and nine genes (41%) in the SP-AI group (**Supplementary Table S3**). The most enriched pathways were the Janus kinase/signal transducers and the activators of transcription (JAK/STAT) signaling (ssc0463). Several other important pathways were also identified, including pathways such as Th17 differentiation (ssc04659), Th1 and Th2 differentiation (ssc04658), and estrogen signaling (ssc04915).

PANTHER GENE ONTOLOGY ANALYSIS

Figure 4 shows (in separate columns) the results of the analyses regarding biological processes and molecular functions using the PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System for gene ontology (GO) (Mi *et al.*, 2019). The results were subdivided by treatment, with the ProxUt, UTJ, and Isth tissues treated with natural mating shown in **Figure 4A**, the semen AI results shown in **Figure 4B**, and the SP-AI treatment results shown in **Figure 4C**. The results of the biological processes after natural mating analysis (**Figure 4A**) showed that downregulated DEGs display biological processes mainly focused on metabolic (GO:0008152) and cellular process (GO:0009987) followed by response to stimulus (GO:0050896). Attending to tissue differences, importance of localization (GO:0051179) and cellular component organization and biogenesis (GO:0071840) was observed in Cvx, DistUt, and ProxUt tissues, while immune system processes (GO:0002376) were only represented in the tissues from UTJ to Inf. In the case of upregulated DEGs, the representation of

biological processes is similar to the response displayed by downregulated genes, but it also seemed to display a particular pattern of metabolic and cellular process in the ProxUt, UTJ, and Isth. In the case of the semen-AI PANTHER results (**Figure 4B**), downregulated genes showed an interesting pattern focused in the Isth, where cellular component organization and biogenesis, and cellular process and localization seem to be particularly promoted. Regarding seminal plasma influence (**Figure 4C**), the data available only found significant biological processes for a few tissues, but upregulated DEGs seemed to display a similar pattern focused on metabolic and cellular processes in the Isth, while downregulated DEGs displayed a range of processes that were largely different to the sperm-containing treatments. Regarding the molecular functions data, binding activity (GO:0005488) seems to be the main function altered in all groups. As well as this, there was an apparent higher transcriptional regulatory activity (GO:0140110) in Amp and Inf tissues, while the catalytic activity (GO:0003824) seems to be more present in Isth than in other tissues, at least in the AI treatments.

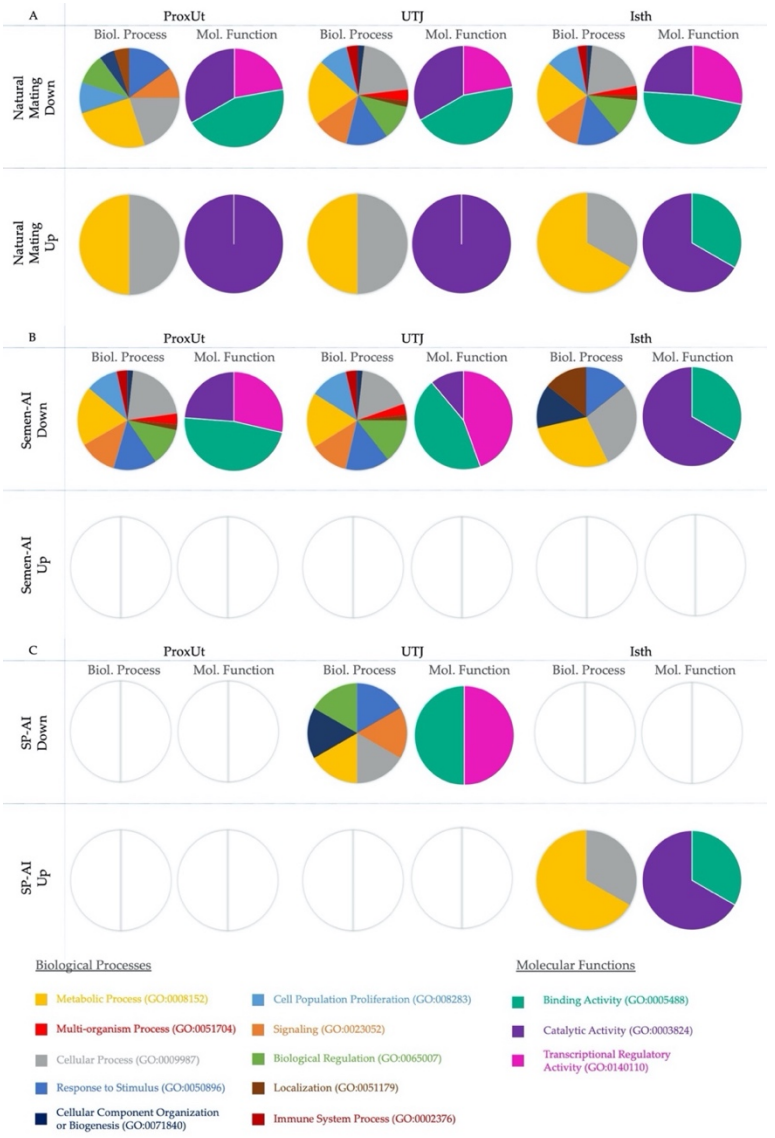

Figure 4. (**A**–**C**) Differentially-expressed genes (DEGs) classified in PANTHER according to biological processes and molecular functions in natural mating (**A**), semen-AI (**B**), and SP-AI (**C**). Rows display the treatment and the up- or downregulation of the analyzed DEGs ($p < 0.05$). Proximal uterus (ProxUt), utero-tubal junction (UTJ), and isthmus (Isth) tissues are shown in the columns. We have specified if the representations correspond to biological processes (Biol. Process; 1st, 3rd, and 5th column) or molecular functions (Mol. Function; 2nd, 4th, and 6th column) are depicted in colors. Empty charts indicate where no matches were found for those DEGs, or if these DEGs were absent from that experimental group.

DISCUSSION

In the present study, we analyzed preovulatory expression changes of the glucocorticoid receptor NR3C1/GR-related genes in tissues collected from the porcine reproductive tract 24 h after natural mating or intra-cervical insemination/infusion of the first portion of sperm-peak ejaculate or its sperm-free seminal plasma. In general, all treatments tested induced gene expression changes, but natural mating clearly induced the most changes in gene expression as compared to the other tested treatments. Insemination of the peak sperm-rich portion (semen-AI) induced a greater response than its seminal plasma-counterpart (SP-AI), presumably due to the effect exerted by the relevant presence of spermatozoa (Rodríguez-Martínez *et al.*, 2009). Almost all genes analyzed (21 out of 22) displayed changes, and more than 60% of the total DEGs for all treatments were achieved by mating. Natural mating might intrinsically display local and systemic effects, as the sensory stimulation produced by penis buffeting affecting the uterus and oviduct motility (Shafik *et al.*, 2006), or the responses after the semen contact the epithelial lining. Mating influences oviduct motility and secretion due to the expression of genes associated with differentiation of oviductal cells, sperm storage, angiogenesis, gamete transport, or the immune system (Parada-Bustamante *et al.*, 2016). Here, natural mating triggered the differential expression of *NR3C1*, but also of genes related to the regulation of GC in the reproductive tract during the preovulatory phase. Natural mating modulates gene expression along the entire reproductive tract, including the functional reservoir, inducing changes in several species (Atikuzzaman *et al.*, 2015; Suarez, 2008, 2016). It also elicits specific responses from the different *NR3C1*-related genes perhaps oriented to the reduction of cortisol (Brandt *et al.*, 2006). This response may be preventing potentially harmful effects on

spermatozoa by the female immune response, thus GCs may promote the development of tolerance towards spermatozoa in the oviduct (Brandt *et al.*, 2006).

It has been suggested that glucocorticoids may take part in the intrauterine regulations during early pregnancy in bovine (Siemieniuch *et al.*, 2010). In ruminants, previous studies indicated a relevant involvement of glucocorticoids in the regulation of uterine (Brooks *et al.*, 2014). To date, the studies in pig are scarce. What is known is that the control of the levels of active cortisol and the interaction with its receptor, the NR3C1 glucocorticoid receptor, are mediated by the expression of *HSD11B1* and *HSD11B2* conforming the so-call pre-receptor ligand metabolism (Gross & Cidlowski, 2008), while the glucocorticoid receptor action is actively regulated by, among other factors, the FKBP genes, *FKBP4* and *FKBP5*.

The potential role for the genes *HSD11B1* and *HSD11B2* is modulating GC levels in the genital tract (Michael *et al.*, 2003). These genes encode for the hydroxysteroid dehydrogenases, responsible for the bidirectional oxidation of cortisol into cortisone (11β-HSD2) and cortisone reduction to active cortisol (11β-HSD1) (Chapman *et al.*, 2013). Natural mating causes upregulation of *HSD11B2* in tissues from isthmus to infundibulum. This may indicate that cortisol transformation into inactive cortisone in the preovulatory pig oviduct is promoted at transcriptomic level. Meanwhile, the gene *HSD11B1,* responsible for cortisone conversion into active cortisol (Chapman *et al.*, 2013; Michael *et al.*, 2003), was downregulated in all reproductive tract segments from the cervix to the isthmus. Interestingly, in pigs, high 11β-HSD1-mediated cortisol production activity has been related to the inhibition of porcine oocyte maturation (Gong *et al.*, 2017; Webb *et al.*, 2008; Yang *et al.*, 1999) in contrast to species such as the cow, where

it appears to be promoted after ovulation (Acosta *et al.*, 2005; Ruiz-Conca *et al.*, 2019; Wang & Harris, 2015) and is also beneficial for oocyte maturation and fertilization, at least *in vitro* (Acosta *et al.*, 2005; da Costa *et al.*, 2016; Tetsuka & Tanakadate, 2019). Furthermore, in equine species, cortisol does not affect oocyte maturation *in vitro* (Scarlet *et al.*, 2017). The direct effect of GCs in mammalian species appears to vary greatly, implying that GC-sensitivity and regulation might be species-specific, and in part driven by diverse ratios of HSD11B2/1 (Gong *et al.*, 2017).

Even though the active cortisol ligand availability is relatively restricted during follicular maturation in part due to the action of HSD11B2 (Whirledge & Cidlowski, 2010), we found the glucocorticoid receptor activated in oviductal tissues. The glucocorticoid receptor gene (*NR3C1*) is a master transcriptional regulator that plays a key role in a large number of vertebrate physiological functions such as stress signaling, immune system, behavior, metabolic activity, and also reproductive events (Whirledge & Cidlowski, 2017). The expression of *NR3C1* has been described in the female reproductive tract in human (Whirledge *et al.*, 2013), ovine (Simmons *et al.*, 2010; Yang *et al.*, 1996), and bovine endometrium and oviduct (Lee *et al.*, 2007, 2009; Siemieniuch *et al.*, 2010). Our results show that *NR3C1* is upregulated by natural mating in the isthmus and infundibulum, while semen-AI only upregulated the gene in the infundibulum, and the SP-AI treatment only upregulated the gene in the isthmus. The fact that the expression in the isthmus is triggered by both natural mating and also by the first portion of the seminal plasma (SP-AI) may indicate an effect of the complex seminal plasma (Rodríguez-Martínez *et al.*, 2005). Glucocorticoid levels are known to increase shortly after ovulation to exert anti-inflammatory actions, helping after the follicle rupture, maintaining of the corpus luteum, and contributing to steroidogenesis, where glucocorticoids stimulate the pregnenolone to

progesterone conversion (Gross & Cidlowski, 2008; Whirledge & Cidlowski, 2010). In that sense, it could be possible that glucocorticoid receptor expression may be promoted after mating for the upcoming events. Previous studies in *NR3C1* knock-out rodents, found an exaggerated inflammatory response, aberrant immunomodulation and immune cell recruitment (Whirledge *et al.*, 2015), even linked to transgenerational effects (Schmidt *et al.*, 2019b) and important reproductive pathologies (Wang & Liu, 2019). Moreover, there is another receptor with a high degree of sequence homology with NR3C1 and a high, but also promiscuous, affinity for glucocorticoid binding, the mineralocorticoid receptor (*NR3C2*) (Richardson *et al.*, 2016). Despite their similarity, both receptors display very different transcriptional and physiological outcomes upon their activation, and we did not find *NR3C2* expression in our study.

On the other hand, the genes *FKBP4* and *FKBP5* are importantly involved in the regulation of NR3C1 signaling (Fries *et al.*, 2017; Hähle *et al.*, 2019). FKBP51 immunophilin (*FKBP5*) and other cofactors are bound when the receptor is cytosol-located and inactive, resulting in a complex (Schiene-Fischer & Yu, 2001; Zannas *et al.*, 2016). After GC binding, FKBP51 is interchanged with FKBP52 (*FKBP4*) (Davies *et al.*, 2002; Wochnik *et al.*, 2005). One of the functions of FKBP52 consists of translocating the complex to the nucleus (Davies *et al.*, 2002), where NR3C1 complex binds to the glucocorticoid response elements, inducing activation or repression of an important number of genes (Sacta *et al.*, 2016; Whirledge & Cidlowski, 2017). As a part of this mechanism of action, NR3C1 also exerts a rapid induction of *FKPB5* transcription, generating an ultra-short negative feedback loop that hinders the translocation to the nucleus (Wochnik *et al.*, 2005), blocking NR3C1-mediated actions (Denny *et al.*, 2000; Ratajczak *et al.*, 2015; Zannas *et al.*, 2016). Thus, while *FKBP5* seems to block the GC action by reducing receptor affinity for the ligands, *FKBP4* increases its ligand affinity, allowing the

translocation of the receptor to the nucleus (Riggs *et al.*, 2003; Wochnik *et al.*, 2005). This may be in agreement, at least at genomic level, with our results in the uterus, UTJ, and isthmus, where we find an upregulation of the *FKBP5* gene and a downregulation of the *FKBP4* gene after mating. The overexpression of *FKBP5* has been mooted as being responsible for the adaptive mechanism developed in New World primates to control their high levels of circulating GC via immunophilins (Scammell *et al.*, 2001). In contrast, the constant downregulation observed in *FKBP4* expression in both sperm-containing treatments may limit the NR3C1 complex translocating to the nucleus, consequently reducing the detrimental effects of immune attack to preovulatory-present spermatozoa. Overall, the putative roles of *FKBP5* in relation to the biological activity of NF-κB (Erlejman *et al.*, 2014; Zgajnar *et al.*, 2019), might also prevent aberrant immune activation that would affect sperm function and survival in the oviduct.

Other genes involved in GC regulation are *PTGS1* and *PTGS2*, encoding for constitutive and inducible prostaglandin synthase enzymes (PTGS1 or COX-1 and PTGS2 or COX-2, respectively), and *PLA2G4B* that encodes for phospholipase A2 that previously transforms membrane phospholipids in arachidonic acid (ARA). This ARA is subsequently converted by PTGS1/PTGS2 into PGH2, a precursor for active prostaglandins (Langenbach *et al.*, 1995; Sugimoto *et al.*, 2015). We found that natural mating downregulated *PTGS2* and *HSD11B1* in the same tissues, from the cervix to the isthmus. *PTGS2* upregulation is linked to 11β-HSD1 stimulation, and *PTGS2*-derived prostaglandins seem to influence cortisol availability to NR3C1 (Simmons *et al.*, 2010). Thus, our results could indicate that natural mating leads to a downregulation of *PTGS2* in the reproductive tract from cervix to isthmus, while upregulating *PTGS1* in some tissues. Recent evidence of a specific *PTGS1-PTGS2* compensation mechanism involving these two genes indicates they may be responsible for prostaglandin

synthesis in reproductive tissues (Li *et al.*, 2018). Also, the observed increase in *PLA24GB* gene expression, responsible for the ARA precursor of prostaglandins, in the ampulla by natural mating as well as by AI-treatments (semen-AI and SP-AI) might be related to the expression of the oviductal phospholipase A2 gene (*PLA2G4B*) close to the fertilization site and may be specifically stimulated by the first seminal plasma portion.

Interestingly, the *NR3C1*-related heat shock proteins (HSPs) were downregulated by both natural mating and AI using sperm-rich fraction components. *HSPA8* (both mating and semen-AI), *HSPA4L* (mating), *HSP90AB1* (mating) and *HSPA4* (semen-AI) expression changes were triggered in exactly the same oviductal tissues (Amp and Inf). Thus, we suggest that the decrease in the expression of these genes could be related to the inflammatory status of the oviduct, since some of these HSPs are related to inflammation and could elicit innate and adaptive proinflammatory immune responses (Geng *et al.*, 2017; Pockley, 2003). In the same way, *HSPA4L* (mating ($q < 0.05$); SP-AI) and *HSP90AB1* (mating) expression, which was upregulated in the distal uterus, could be also related to inflammation, exerted by the mechanical stimulation produced by mating or the AI-catheter.

Our results also indicate the *STAT* genes (Signal Transducer and Activator of Transcription) may be important in the inflammation and also the transcription associated to cortisol action. These genes are present in the JAK/STAT pathway, which is the main signaling route for many cytokines (Rawlings *et al.*, 2004). In particular, *STAT5* has been shown to have important functions in reproduction and also in NR3C1-mediated transcriptional action (Bednorz *et al.*, 2011; Maj & Chelmonska-Soyta, 2007), via active transcription, and T-cell differentiation (Petta *et al.*, 2016). Natural mating downregulated *STAT5A* in almost all tissues (ProxUt,

DistUt and Isth; $q < 0.05$) while *STAT5B* was only modified in the proximal uterus, and only by the sperm treatments (mating and semen-AI). Overall, these results may indicate that *STAT5A* downregulation, could be related to a reduction of the inflammatory response, which may in turn promote tolerance towards spermatozoa in the preovulatory tract, when spermatozoa have not participated in fertilization. Interestingly, a synergy between *STAT5A*, NF-κB and *NR3C1* enhances *TLR2* expression (Petta *et al.*, 2016), inducing an acute inflammatory response in the uterus that may remove sperm by activating polymorphonuclear neutrophils action in order to prepare an adequate implantation environment for the embryo in the female reproductive tract (Akthar *et al.*, 2020; Ezz *et al.*, 2019). Other STAT proteins have been also related to immune and reproductive functions (Choi *et al.*, 2001; Dimitriadis *et al.*, 2007; Maj & Chelmonska-Soyta, 2007). For example, *STAT3* is downregulated in the uterus and upregulated in the ampulla ($q < 0.05$), infundibulum and in UTJ (by seminal plasma only). The downregulation of *STAT3* in the uterus (and UTJ by SP-AI) may be also related to its role in the immune response, which is mediated by IL-6 and IL-10 (Petta *et al.*, 2016), and recently shown to be associated with an increase in embryonic mortality in pigs after embryo transfer (Martinez, Rubér, *et al.*, 2020). In addition, *STAT1* and *STAT2* are key mediators of the innate immune response providing a first-line defense against pathogens (Bluyssen, 2015), are downregulated by natural mating in the cervix and the uterus and might decrease the inflammation produced by semen deposition. *STAT6*, also downregulated after natural mating in the uterus, is consistent with the suppression of the innate immune response that we hypothesize for the rest of the STAT genes in this tissue.

Finally, natural mating produced in the sperm reservoir the same pattern of down (*HSD11B1, PTGS2, FKBP4,* and *STAT5A*) or upregulation (*NR3C1*, *HSD11B2* and *FKBP5*) of genes directly involved in the glucocorticoid action. A

decrease in GCs availability and action could be beneficial in the sperm reservoir. Recent results in humans have found cortisol, testosterone, and other steroid-like molecules compete with progesterone binding to the sperm-membrane receptor α/β hydrolase domain-containing protein 2 (ABHD2) (Miller *et al.*, 2016). This receptor, responsible for removing inhibitors of the cation channel of sperm (CatSper), in an antagonist competition mechanism, consequently inhibiting hyperactivation (Mannowetz *et al.*, 2017). Thus, GCs could be exerting an anti-capacitation effect by preventing premature CatSper activation occurring prior to ovulation, as well as preventing GCs from competing with progesterone, which is necessary for sperm hyperactivation, release from the reservoir oviductal epithelial cells (Machado *et al.*, 2019), and chemotaxis (Oren-Benaroya *et al.*, 2008). Activation of CatSper channels by progesterone, or even prostaglandins, seems to differ among species (Lishko *et al.*, 2011). We have previously shown that *ABHD2* is downregulated in preovulatory UTJ and isthmus, perhaps preventing premature massive capacitation prior to ovulation (Martinez, Alvarez-Rodriguez, *et al.*, 2020).

MATERIALS AND METHODS

ETHICS STATEMENT

Animal handling was performed conforming to current legislation of Sweden (SJVFS 2017:40) and European Community regulation (European Directive 2010/63/EU, 22/09/2010). The experimental research was previously approved by the "Regional Committee for Ethical Approval of Animal Experiments" (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permits no. 75–12 (10/02/2012) and no. ID1400, 02/02/2018).

EXPERIMENTAL DESIGN OF THE STUDY

Sixteen domestic sows (*Sus scrofa domestica*) in the first day of spontaneous estrus were equally distributed in four groups: control (*n* = 4), the animals were infused cervically with 50 mL of Beltsville thawing solution (BTS) protein-free extender (Pursel & Johnson, 1975); natural mating (*n* = 4), each sow was mated to a single boar; or cervical AI of either pools (5 boars) of the ejaculate sperm-rich first portion semen-AI, *n* = 4), or its sperm-free seminal plasma (SP-AI, *n* = 4) (Alvarez-Rodriguez *et al.*, 2019). Tissue segments from the cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp), and infundibulum (Inf) were surgically collected under general anesthesia, 24 h after the treatments, still preovulation, and subjected to gene expression analysis.

ANIMAL MANAGEMENT

Weaned sows (parity 1–3; *n* = 16) and mature boars (9–11 months old; *n* = 5) with good sperm quality (concentration, motility, and morphology) of Swedish Landrace breed were included as previously described (Alvarez-Rodriguez *et al.*, 2019). Spontaneous 2nd estrus post-weaning was manually checked while sows hold snout contact with a boar for standing reflex, upon which they were mated or alternatively intra-cervically inseminated using standard AI-catheters (Minitüb, Munich, Germany).

COLLECTION AND HANDLING OF SEMEN AND TISSUE SAMPLES

Ejaculates were collected weekly in individual fractions using the gloved-hand method and analyzed as previously described (Alvarez-Rodriguez *et al.*, 2019), to

reach a pattern of regular ejaculates depicting >70% sperm motility and with >75% morphologically-normal spermatozoa. The ejaculate sperm-rich first portion (Pursel & Johnson, 1975) was used for the AI-infusion in the semen-AI or SP-AI groups. Seminal plasma (SP-AI) was harvested through double centrifugation (1,500 × *g*/ 10 min) and checked as sperm- and somatic cell-free. All sows were subjected to surgery (mid-laparotomy) under general anesthesia (Pursel & Johnson, 1975) to remove samples from the cervix, uterus and oviduct. All samples were plunged in liquid nitrogen and later stored (–80 °C) until analyzed. Ovaries contained only un-ovulated follicles, in similar numbers among groups (22.30 ± 7.29, mean ± SD) and the condition of pre-ovulation was confirmed by the ratio of circulating estrogen:progesterone in blood plasma (Pursel & Johnson, 1975).

MICROARRAY HYBRIDIZATION AND SCANNING

Total RNA was extracted from tissue segments with Trizol using the protocols described elsewhere (Atikuzzaman *et al.*, 2017). Complementary DNA (cDNA) of each sample was obtained from RNA (250 ng) of each sample by using the GeneChip®Whole Transcript Plus reagent kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer protocol. The hybridization of cDNA mixture of each sample was made using labeled single-strand fragments of cDNA (3.5 µg; 41 µL) and hybridization master mix (109 µL). This cocktail was incubated at 99 °C for 5 min, decreasing the temperature to 45 °C afterwards. The cocktail hybridization mix (130 µL) of each sample was then loaded then on a microarray chip specific for porcine species (GeneChip®Porcine Gene 1.0 ST Array, ThermoFisher Scientific, Sweden) for incubation under rotation (60 revolutions per minute) at 45 °C for 16 h. After being incubated, the hybridized array was unloaded, washed

and stained (GeneChip®Fluidics Station 450, Affymetrix). The array chip was then scanned by using GeneChip®scanner GCS3000.

MICROARRAY DATA ANALYSIS

All data obtained from each array was analyzed with the Transcriptome Analysis Console (TAC; version 4.0) from Affymetrix. Briefly, robust multi-array average (RMA) normalization, computing average intensity values by background adjustment, quantile normalization among arrays, and logarithmic transformation was performed in order to obtain the values of expression of each of the transcripts. Only genes related to the glucocorticoid receptor (NR3C1/GR) were assessed in detail. These 22 genes were reported as related to NR3C1 by using a combination of the internet-based tools. These interaction networks included protein and genetic interactions, pathways, and co-expression. Also, PANTHER (Protein Analysis Through Evolutionary Relationships) classification system for gene ontology (GO) (Mi *et al.*, 2019) of biological process and molecular function was used for the analysis of the functions of the analyzed genes. Graphical illustration of overrepresented GO terms was produced with the Cytoscape v3.0.0 application CluePedia v2.0.3 (Bindea *et al.*, 2009). Statistics of the normalized gene expression were determined using a linear model with an empirical Bayes method implemented in the specific package of Linear Models for Microarray Analysis ("Limma"). The statistical analyses were performed to detect differential expression of transcripts using a Benjamini-Hochberg FDR ($q < 0.05$) and a PCA-based *p*-value correction for type-I errors made with a statistical cut off $p < 0.05$ or FDR $q < 0.05$) (Jollife, 2014), which was completed using ClustVis (BETA) web tool (Metsalu & Vilo, 2015). The principal component analysis clustered, after linear transformation, multivariate data ordered based on the variance. The

prediction ellipses showed that the probability for a new observation from each group would be inside the ellipse (0.05 error). All the redundant or uncharacterized transcripts were excluded to obtain a final database of differentially-expressed genes. The list of genes found to be differentially expressed were then searched for functional pathways using the KEGG database (Kanehisa *et al*., 2019).

CONCLUSIONS

Overall, the results of this study indicate that natural mating seems to produce a differential response in the reproductive tract of the sow, compared to the use of AI, at least in the preovulatory phase. In porcine, it seems clear that, in terms of effects on gene expression, some inherent effects of natural mating on GC regulation genes, could not be mimicked by AI. Moreover, the genes involved in the glucocorticoid receptor control (*FKBP4* and *FKBP5*), cortisol availability (*HSD11B1* and *HSD11B2*) and JAK-STAT signaling (*STAT5A*), exert a collective glucocorticoid-avoiding response that may prevent detrimental effects in the sperm reservoir and help sperm activation and detach close to the time of ovulation.

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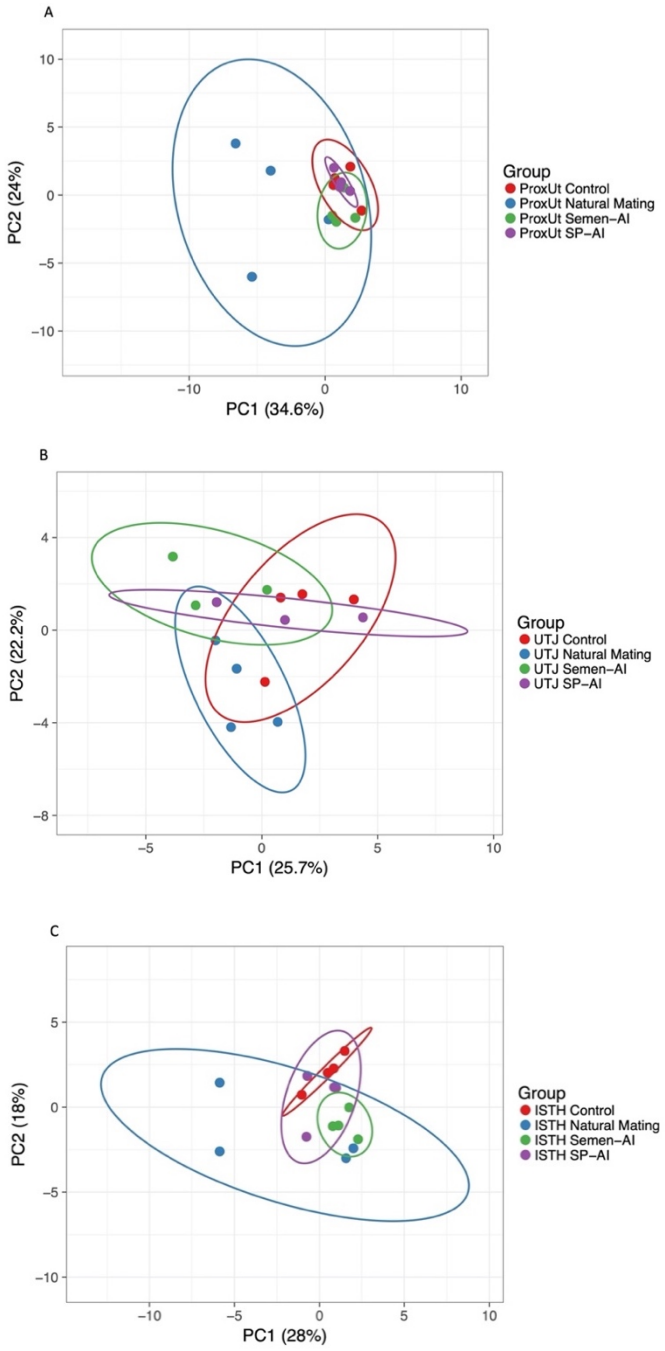
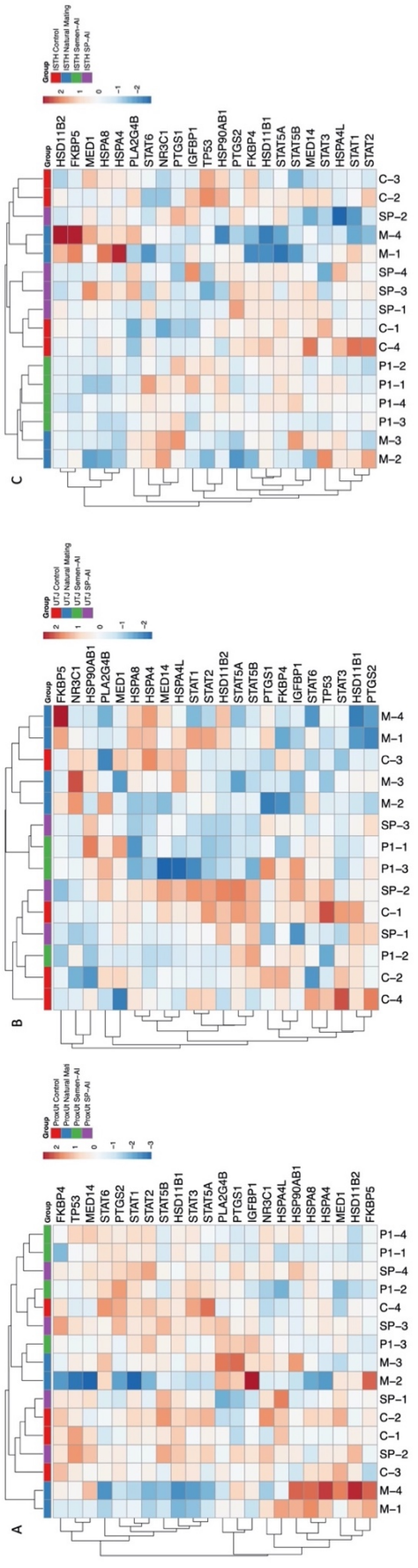
SUPPLEMENTARY MATERIALS

	NATURAL MATING							SEMEN-AI							SP-AI						
	Cvx	DistUt	ProxUt	UTJ	Isth	Amp	Inf	Cvx	DistUt	ProxUt	UTJ	Isth	Amp	Inf	Cvx	DistUt	ProxUt	UTJ	Isth	Amp	Inf
NR3C1					1.3		1.35							1.28					1.35		
HSD11B1	-2.55	**-3.92**	**-3.87**	-2.69	-1.96																
HSD11B2					1.35	1.38	**1.71**			-1.56											
FKBP5		1.5	2.07	1.98	2.36																
FKBP4	-2.22	-1.37	-1.4	**-2**	**-2.73**	**-2.2**	**-1.85**			-1.26		-1.24		-1.34							
PTGS1			1.2			1.3			-1.33							-1.3					
PTGS2	-3.37		-2.06	-2.23	-2.01														1.7		
PLA2G4B						1.34							1.3	1.19						1.29	
IGFBP1																					
HSPA8						-1.26	-1.37				-1.37	-1.52	-1.36	-1.28							
STAT1	-1.66	-1.64	-1.52										-1.26							-1.13	
STAT2	-1.69	-1.67	-1.65																		
STAT3		-1.56	-1.92			**1.44**	1.26											-1.47			
STAT5A	-1.43	**-2.5**	**-2.38**	-1.5	**-1.5**		-1.28														-1.33
STAT5B			-1.3							-1.31											
STAT6		-1.23				1.31	**1.39**						1.26								
TP53	-1.17				-1.17														-1.29		-1.37
MED1						-1.13							-1.16								
MED14						-1.1	-1.21														
HSPA4	-1.45												-1.34	-1.21							
HSPA4L		**1.58**				-1.67	-2.02	-2.25			-1.48					1.8					
HSP90AB1		1.18				-1.26	-1.17														

Supplementary Figure 1. Differentially expressed genes (DEGs) of cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf), ordered by treatments (natural mating, semen-AI or SP-AI). Numbers represent the fold change for each gene on each tissue, compared to control. Upregulated genes ($p < 0.05$) are marked in green, while downregulated genes ($p < 0.05$) are shown in red colour. Colour grading is displayed ranging from 1 (upregulated), and from -1 (downregulated) in every treatment, separately. FDRs ($q < 0.05$) is noted in bold.



Supplementary Figure 2. Principal Component Analysis (PCA) of **A**) proximal uterus (ProxUt), **B**) utero-tubal junction (UTJ) and **C**) isthmus (Isth) was depicted. The prediction ellipses show the probability for a new observation from each group will be inside the ellipse (0.05 error). Principal component 1 (PC1) and principal component 2 (PC2) explain percentage of the total variance, respectively.



Supplementary Figure 3. Heat map of **A**) proximal uterus (ProxUt), **B**) utero-tubal junction (UTJ) and **C**) isthmus (Isth) was depicted. Data was clustered using correlation distance and average linkage.

Supplementary Table 1. Kyoto Encyclopedia of Gens and Genomes (KEGG) Pathways of genes differentially expressed by natural mating. Upregulation is represented in bold and downregulation in non-bold ($p < 0.05$) in each tissue: cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf). Also, false discovery rates (FDRs) are shown in red ($q < 0.05$).

Natural Mating		
Gene List	**Tissue**	**Pathway Name & ID**
NR3C1	**Isth, Inf**	Neuroactive ligand-receptor interaction (ssc04080)
HSD11B1	Cvx, DistUt, ProxUt, UTJ, Isth	Steroid hormone biosynthesis (ssc00140) Metabolism of xenobiotics by cytochrome P450 (ssc00980) Metabolic pathways (ssc01100)
HSD11B2	**Isth, Amp, Inf**	Steroid hormone biosynthesis (ssc00140) Metabolic pathways (ssc01100) Aldosterone-regulated sodium reabsorption (ssc04960)
FKBP5	**DistUt, ProxUt, UTJ, Isth**	Estrogen signaling pathway (ssc04915)
PTGS1	**ProxUt, Amp**	Arachidonic acid metabolism (ssc00590) Metabolic pathways (ssc01100) Platelet activation (ssc04611) Serotonergic synapse (ssc04726) Regulation of lipolysis in adipocytes (ssc04923)
PTGS2	Cvx, DistUt, UTJ, **Isth**	Arachidonic acid metabolism (ssc00590) Metabolic pathways (ssc01100) NF-kappa B signaling pathway (ssc04064) VEGF signaling pathway (ssc04370) C-type lectin receptor signaling pathway (ssc04625) IL-17 signaling pathway (04657) TNF signaling pathway (ssc04668) Retrograde endocannabinoid signaling (ssc04723) Serotonergic synapse (ssc04726) Ovarian steroidogenesis (ssc04913) Oxytocin signaling pathway (ssc04921) Regulation of lipolysis in adipocytes (ssc04923)

PLA2G4B	Amp	Glycerophospholipid metabolism (ssc00564) Ether lipid metabolism (ssc00565) Arachidonic acid metabolism (ssc00590) Linoleic acid metabolism (ssc00591) alpha-Linolenic acid metabolism (ssc00592) Metabolic pathways (ssc01100) MAPK signaling pathway (ssc04010) Ras signaling pathway (ssc04014) Phospholipase D signaling pathway (ssc04072) Necroptosis (ssc04217) Vascular smooth muscle contraction (ssc04270) VEGF signaling pathway (ssc04370) Platelet activation (ssc04611) Fc epsilon RI signaling pathway (ssc04664) Fc gamma R-mediated phagocytosis (ssc04666) Glutamatergic synapse (ssc04724) Serotonergic synapse (ssc04726) Long-term depression (ssc04730) Inflammatory mediator regulation of TRP channels (ssc04750) GnRH signaling pathway (ssc04912) Ovarian steroidogenesis (ssc04913) Oxytocin signaling pathway (ssc04921) Choline metabolism in cancer (ssc05231)
HSPA8	Amp, Inf	Spliceosome (ssc03040) MAPK signaling pathway (ssc04010) Protein processing in endoplasmic reticulum (ssc04141) Endocytosis (ssc04144) Longevity regulating pathway - multiple species (ssc04213) Antigen processing and presentation (ssc04612) Estrogen signaling pathway (ssc04915)
FKBP4	Cvx, DistUt, ProxUt, UTJ, Isth, Amp, Inf	Estrogen signaling pathway (ssc04915)
STAT1	Cvx, DistUt, ProxUt	Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) Toll-like receptor signaling pathway (ssc04620) NOD-like receptor signaling pathway (ssc04621) C-type lectin receptor signaling pathway (ssc04625) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) Thyroid hormone signaling pathway (ssc04919) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT2	Cvx, DistUt, ProxUt	Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) NOD-like receptor signaling pathway (ssc04621) C-type lectin receptor signaling pathway (ssc04625) Jak-STAT signaling pathway (ssc04630)

STAT3	DistUt, ProxUt, **Amp**, **Inf**	EGFR tyrosine kinase inhibitor resistance (ssc01521) Chemokine signaling pathway (ssc04062) HIF-1 signaling pathway (ssc04066) FoxO signaling pathway (ssc04068) Necroptosis (ssc04217) Signaling pathways regulating pluripotency of stem cells (ssc04550) Jak-STAT signaling pathway (ssc04630) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) Adipocytokine signaling pathway (ssc04920) Insulin resistance (ssc04931) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT5A	Cvx, DistUt, ProxUt, UTJ, Isth, Inf	ErbB signaling pathway (ssc04012) Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT5B	**ProxUt**	ErbB signaling pathway (ssc04012) Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT6	DistUt, **Amp**, **Inf**	Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659)

TP53	Cvx, Isth	Endocrine resistance (ssc01522) Platinum drug resistance (ssc01524) MAPK signaling pathway (ssc04010) Sphingolipid signaling pathway (ssc04071) Cell cycle (ssc04110) p53 signaling pathway (ssc04115) Mitophagy – animal (ssc04137) PI3K-Akt signaling pathway (ssc04151) Apoptosis (ssc04210) Longevity regulating pathway (ssc04211) Ferroptosis (ssc04216) Cellular senescence (ssc04218) Wnt signaling pathway (ssc04310) Neurotrophin signaling pathway (ssc04722) Thyroid hormone signaling pathway (ssc04919)
MED1	Amp	Endocrine resistance (ssc01522) Thyroid hormone signaling pathway (ssc04919)
MED14	Amp, Inf	Thyroid hormone signaling pathway (ssc04919)
HSPA4	Cvx	Tight junction (ssc04530) Antigen processing and presentation (ssc04612)
HSPA4L	DistUt, Amp, Inf	Protein processing in endoplasmic reticulum (ssc04141)
HSP90AB1	DistUt, Amp, Inf	Protein processing in endoplasmic reticulum (ssc04141) PI3K-Akt signaling pathway (ssc04151) Necroptosis (ssc04217) Antigen processing and presentation (ssc04612) NOD-like receptor signaling pathway (ssc04621) IL-17 signaling pathway (ssc04657) Th17 cell differentiation (ssc04659) Progesterone-mediated oocyte maturation (ssc04914) Estrogen signaling pathway (ssc04915)

Supplementary Table 2. KEGG Pathways of genes differentially expressed by cervical insemination of the first portion of the sperm-rich ejaculate fraction (semen-AI). Upregulation is shown in bold and downregulation in non-bold ($p < 0.05$) in each tissue: cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf).

Semen-AI		
Gene List	**Tissue**	**Pathway Name & ID**
NR3C1	**Inf**	Neuroactive ligand-receptor interaction (ssc04080)
HSD11B2	ProxUt	Steroid hormone biosynthesis (ssc00140) Metabolic pathways (ssc01100) Aldosterone-regulated sodium reabsorption (ssc04960)
PTGS1	DistUt	Arachidonic acid metabolism (ssc00590) Metabolic pathways (ssc01100) Platelet activation (ssc04611) Serotonergic synapse (ssc04726) Regulation of lipolysis in adipocytes (ssc04923)
PLA2G4B	**Amp, Inf**	Glycerophospholipid metabolism (ssc00564) Ether lipid metabolism (ssc00565) Arachidonic acid metabolism (ssc00590) Linoleic acid metabolism (ssc00591) alpha-Linolenic acid metabolism (ssc00592) Metabolic pathways (ssc01100) MAPK signaling pathway (ssc04010) Ras signaling pathway (ssc04014) Phospholipase D signaling pathway (ssc04072) Necroptosis (ssc04217) Vascular smooth muscle contraction (ssc04270) VEGF signaling pathway (ssc04370) Platelet activation (ssc04611) Fc epsilon RI signaling pathway (ssc04664) Fc gamma R-mediated phagocytosis (ssc04666) Glutamatergic synapse (ssc04724) Serotonergic synapse (ssc04726) Long-term depression (ssc04730) Inflammatory mediator regulation of TRP channels (ssc04750) GnRH signaling pathway (ssc04912) Ovarian steroidogenesis (ssc04913) Oxytocin signaling pathway (ssc04921) Choline metabolism in cancer (ssc05231)

HSPA8	UTJ, Isth, Amp, Inf	Spliceosome (ssc03040) MAPK signaling pathway (ssc04010) Protein processing in endoplasmic reticulum (ssc04141) Endocytosis (ssc04144) Longevity regulating pathway - multiple species (ssc04213) Antigen processing and presentation (ssc04612) Estrogen signaling pathway (ssc04915)
FKBP4	ProxUt, Isth, Inf	Estrogen signaling pathway (ssc04915)
STAT1	Amp	Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) Toll-like receptor signaling pathway (ssc04620) NOD-like receptor signaling pathway (ssc04621) C-type lectin receptor signaling pathway (ssc04625) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) Thyroid hormone signaling pathway (ssc04919)AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT5B	ProxUt	ErbB signaling pathway (ssc04012) Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT6	**Amp**	Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659)
MED1	Amp	Endocrine resistance (ssc01522) Thyroid hormone signaling pathway (ssc04919)
HSPA4	Amp, Inf	Tight junction (ssc04530) Antigen processing and presentation (ssc04612)
HSPA4L	Cvx, UTJ	Protein processing in endoplasmic reticulum (ssc04141)

Supplementary Table 3. KEGG Pathways of genes differentially expressed by cervical insemination of the sperm-free seminal plasma of the first portion of the sperm-rich fraction (SP-AI). Upregulation is shown in bold and downregulation in non-bold ($p < 0.05$) in each tissue: cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf).

SP-AI		
Gene List	**Tissue**	**Pathway Name & ID**
NR3C1	**Isth**	Neuroactive ligand-receptor interaction (ssc04080)
PTGS1	DistUt	Arachidonic acid metabolism (ssc00590) Metabolic pathways (ssc01100) Platelet activation (ssc04611) Serotonergic synapse (ssc04726) Regulation of lipolysis in adipocytes (ssc04923)
PTGS2	**Isth**	Arachidonic acid metabolism (ssc00590) Metabolic pathways (ssc01100) NF-kappa B signaling pathway (ssc04064) VEGF signaling pathway (ssc04370) C-type lectin receptor signaling pathway (ssc04625) IL-17 signaling pathway (04657) TNF signaling pathway (ssc04668) Retrograde endocannabinoid signaling (ssc04723) Serotonergic synapse (ssc04726) Ovarian steroidogenesis (ssc04913) Oxytocin signaling pathway (ssc04921) Regulation of lipolysis in adipocytes (ssc04923)
PLA2G4B	**Amp**	Glycerophospholipid metabolism (ssc00564) Ether lipid metabolism (ssc00565) Arachidonic acid metabolism (ssc00590) Linoleic acid metabolism (ssc00591) alpha-Linolenic acid metabolism (ssc00592) Metabolic pathways (ssc01100) MAPK signaling pathway (ssc04010) Ras signaling pathway (ssc04014) Phospholipase D signaling pathway (ssc04072) Necroptosis (ssc04217) Vascular smooth muscle contraction (ssc04270) VEGF signaling pathway (ssc04370) Platelet activation (ssc04611) Fc epsilon RI signaling pathway (ssc04664) Fc gamma R-mediated phagocytosis (ssc04666) Glutamatergic synapse (ssc04724) Serotonergic synapse (ssc04726)

		Long-term depression (ssc04730) Inflammatory mediator regulation of TRP channels (ssc04750) GnRH signaling pathway (ssc04912) Ovarian steroidogenesis (ssc04913) Oxytocin signaling pathway (ssc04921) Choline metabolism in cancer (ssc05231)
STAT1	Amp	Chemokine signaling pathway (ssc04062) Necroptosis (ssc04217) Toll-like receptor signaling pathway (ssc04620) NOD-like receptor signaling pathway (ssc04621) C-type lectin receptor signaling pathway (ssc04625) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) Thyroid hormone signaling pathway (ssc04919)
STAT3	UTJ	EGFR tyrosine kinase inhibitor resistance (ssc01521) Chemokine signaling pathway (ssc04062) HIF-1 signaling pathway (ssc04066) FoxO signaling pathway (ssc04068) Necroptosis (ssc04217) Signaling pathways regulating pluripotency of stem cells (ssc04550) Jak-STAT signaling pathway (ssc04630) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) Adipocytokine signaling pathway (ssc04920) Insulin resistance (ssc04931) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)
STAT5A	Inf	ErbB signaling pathway (ssc04012) Necroptosis (ssc04217) Jak-STAT signaling pathway (ssc04630) Th1 and Th2 cell differentiation (ssc04658) Th17 cell differentiation (ssc04659) Prolactin signaling pathway (ssc04917) AGE-RAGE signaling pathway in diabetic complications (ssc04933) Growth hormone synthesis, secretion and action (ssc04935)

TP53	Isth, Inf	Endocrine resistance (ssc01522) Platinum drug resistance (ssc01524) MAPK signaling pathway (ssc04010) Sphingolipid signaling pathway (ssc04071) Cell cycle (ssc04110) p53 signaling pathway (ssc04115) Mitophagy – animal (ssc04137) PI3K-Akt signaling pathway (ssc04151) Apoptosis (ssc04210) Longevity regulating pathway (ssc04211) Ferroptosis (ssc04216) Cellular senescence (ssc04218) Wnt signaling pathway (ssc04310) Neurotrophin signaling pathway (ssc04722) Thyroid hormone signaling pathway (ssc04919)
HSPA4L	DistUt	Protein processing in endoplasmic reticulum (ssc04141)

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CHAPTER V

Seminal Plasma Triggers the Differential Expression of the Glucocorticoid Receptor (*NR3C1/GR)* in the Rabbit Reproductive Tract

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SIMPLE SUMMARY

Glucocorticoids are steroid hormones modulating different functions in mammals, including reproduction, that act through the glucocorticoid receptor, encoded by the gene called *NR3C1*. Here, we describe how the expression levels of the glucocorticoid receptor change along the different compartments of the female rabbit internal reproductive tract 20 h after insemination with sperm-free seminal plasma or natural mating (whole semen) (*Experiment 1*); and how these levels change over time at 10, 24, 36, 68, and 72 h post-mating, during specific reproductive stages, i.e., ovulation, fertilization and the interval of early embryo development to the morula stage occurs (*Experiment 2*). *NR3C1*-upregulation was found in the infundibulum at 20 h after all treatments, especially after sperm-free seminal plasma infusion compared to mating (*Experiment 1*). In *Experiment 2*, the receptor gene expression levels increased in a spatio-temporal sequence, corresponding to the assumed location of the rabbit embryos (particularly morulae) in the oviductal various segments and timepoints (particularly 72 h), compared to down-expression at uterine regions. We conclude that *NR3C1* may play a relevant role in the rabbit female reproductive tract.

ABSTRACT

Rabbits are interesting as research animal models for reproduction, due to their condition of species of induced ovulation, with the release of endogenous gonadotropin-releasing hormone (GnRH) due to coitus. Glucocorticoid (GC) signaling, crucial for physiological homeostasis, is mediated through a yet unclear mechanism, by the GC receptor (NR3C1/GR). After mating, the female reproductive tract undergoes dynamic modifications, triggered by gene transcription, a pre-amble for fertilization and pregnancy. This study tested the hypothesis that when ovulation is induced, the expression of *NR3C1* is influenced by sperm-free seminal plasma (SP), similarly to what occurs after mating (whole semen), along the different segments of the internal reproductive tract of female rabbits. Semen (mating) was compared to vaginal infusion of sperm-free SP (*Experiment 1*), and changes over time were also evaluated, i.e., 10, 24, 36, 68, and 72 h post-mating, corresponding to specific stages, i.e., ovulation, fertilization, and the interval of early embryo development up to the morula stage (*Experiment 2*). All does were treated with GnRH to induce ovulation. Samples were retrieved from seven segments of the reproductive tract (from the cervix to infundibulum), at 20 h post-mating or sperm-free SP infusion (*Experiment 1*) or at 10, 24, 36, 68, and 72 h post-mating (*Experiment 2*). Gene expression of *NR3C1* was analyzed by qPCR. Results showed an increase in *NR3C1* expression in the infundibulum compared to the other anatomical regions in the absence of spermatozoa when sperm-free SP infusion was performed (*Experiment 1*). Moreover, during the embryo transport through the oviduct, the distal isthmus was time-course upregulated, especially at 72 h, when morulae are retained in this anatomical region, while it was downregulated in the distal uterus at 68 h (*Experiment 2*). The overall results suggest that *NR3C1*, the

GC receptor gene, assessed in the reproductive tract of does for the first time, shows differential expression changes during the interval of oviductal and uterine embryo transport that may imply a relevant role of the GC action, not only close to the site of ovulation and fertilization, but also in the endometrium.

Keywords: glucocorticoid receptor; gene expression; RT-qPCR; seminal plasma; female genital tract; rabbit.

INTRODUCTION

Rabbits (*Oryctolagus cuniculus*) are wild animals originally from the south of Europe, which were domesticated and widely introduced around the world, nowadays present in every continent except Antarctica (Tablado *et al.*, 2009), and even considered as a pest in some areas (Roy-Dufresne *et al.*, 2019). These animals have been historically consumed in Mediterranean countries (Petraci & Cavani, 2013), while recently becoming important as production animals in some other regions (Li *et al.*, 2018). They are also particularly well suited as model organisms for basic and applied reproductive experimental research, for their similarity to the chronology of human early embryonic development (Fischer *et al.*, 2012; Püschel *et al.*, 2010) and, especially, as a species characterized for copulation-induced ovulation as some felids (Jorge-Neto *et al.*, 2020) or camelids (Mauricio Silva *et al.*, 2020), best suited for experimental studies due to its early sexual maturation, short gestation, prolificacy and small size (Adams *et al.*, 2016). The use of assisted reproduction techniques (ARTs) in induced-ovulators is constrained by endocrine imbalances affecting the crucial steps of fertilization and early embryo development (Pelican *et al.*, 2006). Therefore, the rabbit is an interesting animal model to improve intracytoplasmic sperm injection, embryo culture, embryo transfer, or cryopreservation (Garcia-Dominguez *et al.*, 2019), but also for commercial artificial insemination (AI) in rabbit intensive meat production (Piles *et al.*, 2013).

Being an induced-ovulator, the rabbit requires the generation of genital-somatosensory signals during coitus to activate midbrain and brainstem noradrenergic neurons and generate the preovulatory peak of gonadotropin-releasing hormone (GnRH) (Bakker & Baum, 2000; Ratto *et al.*, 2019). The efficiency of natural mating, and also ARTs, relies on factors encompassing male

and female parameters (Brun *et al.*, 2016) and the interaction of both (Parada-Bustamante *et al.*, 2016; Robertson, 2005), hence improving the understanding of the effect of these factors in rabbit early development may improve fertility outcomes.

Although the role of ovarian steroid hormones progesterone and estrogens in the signaling pathways of reproductive stages is well-known, the importance of glucocorticoids (GCs) as regulators of reproduction is starting to be more recognized (Whirledge *et al.*, 2017; Whirledge & Cidlowski, 2017). GCs are steroid hormones under the control of the hypothalamic–pituitary–adrenal axis, which are crucial for stress responses, behavior, and reproduction in mammals (Wang & Harris, 2015). Even though GC production is essential for adequate physiology, they have commonly been assumed to be detrimental to reproductive performance and fertility, regarding the link between high GC levels and chronic stress (Whirledge & Cidlowski, 2013). However, GC basal levels have an important role in reproduction, comprising important reproductive events such as male–female mating interaction, oocyte maturation, early embryo development, fetus–mother communication, parturition, and lactation (Whirledge & Cidlowski, 2010; Whirledge & Cidlowski, 2017). Although the underlying action mechanism of these hormones is complex and still not sufficiently understood, the GC receptor (NR3C1/GR) is assumed to play a key function in the mediation of GC action and gene transcription (Bekhbat *et al.*, 2017; Cain & Cidlowski, 2017; Ratman *et al.*, 2013), including reproduction and embryo development (Ruiz-Conca, Gardela, Alvarez-Rodríguez, *et al.*, 2019; Whirledge *et al.*, 2015; Whirledge & Cidlowski, 2017; Yang *et al.*, 1999). The GCs bind to their receptor constituting a complex that can be transported to the nucleus, where they bind to GC response elements (GRE) of the DNA sequence, inducing the activation or repression of gene transcription (Petta *et al.*, 2016; Wochnik *et al.*, 2005), which can thereby modulate

the changing female environment during the early embryo development stage (Majewska *et al.*, 2012; Siemieniuch *et al.*, 2010; Simmons *et al.*, 2010; Whirledge *et al.*, 2015). Thus, GCs have been shown to influence the female reproductive tract, including the prostaglandin-mediated smooth muscle contractility movements (Lindblom *et al.*, 1980; Wånggren *et al.*, 2008), the corpus luteum formation and function (Andersen, 2002), the Janus kinase/signal transducers and the activators of transcription (JAK/STAT) pathway, the immune response or the estrogen signaling, among others (Ruiz-Conca *et al.*, 2020). The effects of GC exposure to oocytes and preimplantational embryos are still not completely known, as whether they have a protective, innocuous, or harmful effect seems to greatly differ among mammalian species (da Costa *et al.*, 2016; Gong *et al.*, 2017; Ruiz-Conca *et al.*, 2019; Scarlet *et al.*, 2017). In rabbits, *NR3C1* has been recently postulated as a candidate gene implicated in reproductive seasonal differences between wild and domestic animals (Carneiro *et al.*, 2015).

Since understanding the role of *NR3C1* in the reproductive tract is relevant for reproductive biology, we attempted to describe the GC receptor expression (*NR3C1*) of organ samples collected along the different anatomical segments of female rabbits internal reproductive tract, in response to natural mating or sperm-free seminal plasma (SP) infusion for the purpose of determining whether sperm-free SP was able to specifically affect gene expression similarly to mating, and how mating-induced *NR3C1* expression changes over time during the interval of early embryo development (10 h to 72 h).

MATERIALS AND METHODS

ETHICS STATEMENT

The handling of the animals was performed according to the standards of animal care according to the Spanish Law (RD1201/2005), the European Directive (2010/63/EU; (BOE, 2005: 252:34367-91)) and the Directive 2010/63/EU of the European Parliament and of the Council of 22th September 2010 on the protection of animals used for scientific purposes (2010; 276:33-79). The Committee of Ethics and Animal Welfare of the Universitat Autònoma de Barcelona (Spain) approved this study (Expedient #517).

ANIMALS

Adult New Zealand White rabbit bucks (n = 6) and does (n = 24), from 7 to 13 months old (mo), coming from an experimental farm of the Institut de Recerca i Tecnologia Agroalimentaries (IRTA, Torre Marimon, Spain) were used in this study. The animals were housed in individual cages for each rabbit (85 × 40 × 30 cm) equipped with plastic footrests, a feeder (restricted to 180 g/day of an all-mash pellet) and a nipple drinker (fresh water was always available). The environmental conditions were controlled, with a 16 h/8 h light/darkness photoperiod, temperature ranging from 15 to 20 °C during winter and from 20 to 26 °C during summer, and relative humidity between 60% to 75% was maintained by a forced ventilation system.

For the obtention of the ejaculate, the males were trained using an artificial vagina when they were 4.5 mo. A homemade polyvinyl chloride artificial vagina, containing water at 50 °C, was used. For this study, only one ejaculate per male

was collected, discarding the ejaculates containing urine and/or calcium carbonate deposits.

EXPERIMENTAL DESIGN

The experimental design used in this study is based on our previously described experimental approach (Gardela *et al.*, 2020).

EXPERIMENT 1: ANALYSIS OF GENE EXPRESSION DIFFERENCES IN THE DOES' REPRODUCTIVE TRACT, AT 20 H POST-MATING (WHOLE SEMEN) OR SEMINAL PLASMA (SP) INFUSION (SPERM-FREE)

Gene expression analyses for *NR3C1* were performed in sequential segments of the female reproductive tracts (n = 9): endocervix (Cvx), endometrium (distal uterus, DistUt; proximal uterus, ProxUt), utero-tubal junction (UTJ), distal isthmus (Isth), ampulla (Amp), and infundibulum (Inf). Tissue samples were collected at 20 h post-treatment with 0.03 mg GnRH im (intramuscular; Fertagyl®, Esteve Veterinaria, Barcelona, Spain) in all experimental groups (n = 9): post-mating (n = 3), post-SP-infusion (n = 3) and control, no mating or infusion, (n = 3, control group).

EXPERIMENT 2: ANALYSIS OF GENE EXPRESSION DIFFERENCES IN THE REPRODUCTIVE TRACT OF MATED RABBIT FEMALES FROM 10 H POST-MATING TO UP TO 72 H POST-MATING

A group of 15 rabbit does were sequentially euthanized at 10, 24, 36, 68, and 72 h post-mating (n = 3, time of collection). Reproductive tract sections (Cvx, DistUt, ProxUt, UTJ, Isth, Amp, and Inf) were recovered for gene expression analysis

for *NR3C1*. The 10 h post-mating group was established as the reference group as this is the presumed time of ovulation in rabbits.

MATING AND SEMEN COLLECTION

The rabbit does included in the mating groups of *Experiment 1* and *2* were sequentially mated with two randomly selected bucks to decrease male-variation effects. The does additionally received an injection of 0.03 mg GnRH im previously to being mated to reinforce ovulation. Ovulation is expected at about 10 h after GnRH stimulation in all groups. After the semen collection from the same rabbit bucks, as described above, the sperm-free SP was isolated after centrifugation at 2000× *g* for 10 min and checked for the absence of spermatozoa. The harvested sperm-free SP was immediately pooled for vaginal infusions of *Experiment 1*.

COLLECTION OF TISSUES AND EMBRYOS

The does were euthanized by the administration of 600 mg pentobarbital sodium (Dolethal, Vetoquinol, Madrid, Spain) intravenously (marginal ear vein). Then, the samples of the female reproductive tracts were randomly chosen from the same lateral side (right), segmented and collected. The tissues of the oviductal segments were collected in toto. In *Experiment 2*, before segment-sectioning the internal reproductive tract, the entire oviduct was isolated from the uterus. In mated does, embryos were collected by flushing the oviduct (phosphate buffer saline supplemented with 5% fetal calf serum and 1% antibiotic–antimycotic solution), which were examined by number and developmental stage. The number of ovarian follicles and the number of embryos on each developmental stage were annotated and have been published elsewhere (Gardela *et al.*, 2020).

Briefly, at 24 h, 53.0 ± 40.2% of the embryos (2-4 cell stage) were recovered, at 36 h the embryo recovery rate was 84.1 ± 31.5% (8-cell stage), at 68 h it was 103.7 ± 17.1% (morula), and at 72 h, 104.8 ± 6.7% of the embryos (compacted morula) were retrieved, with respect to the total of ovulated follicles counted at each stage (Mean ± SD). Intervals of embryo development in the mated group were extrapolated to the sperm-free SP-infused does. All reproductive segments were stored in RNAlater solution at −80 °C.

REAL TIME QUANTITATIVE PCR ANALYSES

The TRIzol-based protocol was used for the total RNA extraction, as described elsewhere (Gardela *et al.*, 2020). Briefly, in 1 mL TRIzol was used to mechanically disrupt the tissues (TissueLyser II with 7 mm stainless steel beads, Qiagen, Germany). The homogenized tissues underwent different centrifugation steps and were incubated with isopropanol and RNA precipitation solution (1.2 M NaCl and 0.8 M $Na_2C_6H_6O_7$) for RNA pellet obtaining. The RNA concentration and quality were determined from the absorbance of 260 nm measured by Thermo Scientific NanoDrop™ 2000, and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively. The first strand cDNA synthesis was performed using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Foster City, CA, USA) and the samples were stored at −20 °C until further analyses. Quantitative PCR (qPCR) was performed in a Real-Time PCR Detection System (CFX96™; Bio-Rad Laboratories, Inc; Hercules, CA, USA) following the steps previously described (Gardela *et al.*, 2020). Two technical replicates were used for each sample. **Figure S1** depicts the melting curves and the peak curves of *β-ACTIN* and *NR3C1*. Efficiencies of the primers were calculated using five different concentrations of the same cDNA sample (serial dilutions of 1/5), using three

technical replicates for each concentration. The gene relative expression levels were quantified using the Pfaffl method (Pfaffl, 2001) and β-*ACTIN* as a housekeeping gene for cDNA normalization. The primer sequences, product sizes, and efficiencies are shown in **Table 1**. For the β-*ACTIN* gene, commercial gene-specific PCR primers for rabbit were used (PrimePCR™ SYBR® Green Assay: ACTB, Rabbit; Bio-Rad Laboratories, Inc; Hercules, CA, USA). The amplicons of qPCR were loaded into an agarose gel after mixing with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) to confirm the product sizes (**Figure S2**). After running, the gel was imaged by a gel imaging system (ChemiDoc XRS+ System, BioRad Laboratories, Inc; Hercules, CA, USA).

Table 1. Primers used for the quantitative PCR analyses.

Gene	Primer Sequence (5′-3′)	Product Size (bp)	Efficiency (%)
NR3C1	F: CACAACTCACCCCAACACTG	212	89.6
	R: CAGGAGGGTCATTTGGTCAT		
β-*ACTIN*	F: commercial, not available	120	88.6
	R: commercial, not available		

NR3C1: glucocorticoid receptor; *β-ACTIN:* beta-actin. F: forward, R: reverse, A: adenine, C: cytosine, G: guanine, T: thymine, bp: base pair.

STATISTICAL ANALYSES

All data were processed with CFX Maestro™ 1.1 software version 4.1.2433.1219 (Bio-Rad Laboratories, Inc; Hercules, CA, USA) and were analyzed for normal distribution and homoscedasticity using the Shapiro–Wilk Normality test and Levene's test. Log(x) transformation was used to restore a normal distribution prior to analysis. The statistical analysis was conducted in R version 3.6.1. (R Core Team, 2019) with *nlme* (Pinheiro *et al.*, 2021) to perform linear mixed-effects (LME)

models and *multcomp* (Hothorn *et al.*, 2008) to perform pairwise comparisons adjusted by Tukey's test. Data are presented as median (minimum, maximum), unless otherwise stated. The threshold for significance was set at $p < 0.05$.

Treatments of the *Experiment 1* (negative control, mating (positive control) and sperm-free SP) were used as fixed effects and each individual doe as the random part of the model. Pairwise comparisons were adjusted by Tukey's test. A second LME model was used including the different sample collection times of *Experiment 2* (10, 24, 36, 68, and 72 h post-mating) as fixed effects and each individual doe as the random part of the model. Post-hoc comparisons were performed using Tukey's multiple comparisons test.

Finally, the differential expression changes in qPCR results among anatomical segments (Cvx to Inf) in the *Experiment 1* and *2* were further re-analyzed, using the UTJ as an arbitrary reference anatomical medial compartment among all samples examined, which is located in the middle of the tract. This was performed in order to compare gene expression changes, per gene, issued both by mating or sperm-free SP vaginal infusion, respectively, to control (*Experiment 1*) or by different times post-mating: 10, 24, 36, 68, and 72 h (*Experiment 2*), among the different tissues of the female reproductive tract. Each tissue was included as fixed effects and each individual doe as the random part of the LME model. As stated above, the analysis of differences among each tissue of the female reproductive tract was performed by the Tukey's multiple comparison test.

RESULTS

EXPERIMENT 1: DIFFERENTIAL GENE EXPRESSION IN RABBIT FEMALE REPRODUCTIVE TRACT AT 20 H AFTER NATURAL MATING OR INFUSION OF SPERM-FREE SEMINAL PLASMA

The results of *Experiment 1* are shown in **Figure 1**, where differential expression changes in *NR3C1* in the different anatomical segments of the rabbit reproductive tract were analyzed 20 h after mating or SP infusion. First, expression changes in each segment were compared between the negative control and the treatments of natural mating and SP-infusion. Significative differences in *NR3C1* expression between natural mating and SP infusion were shown in Inf ($p < 0.05$) (**G**), where the sperm-free SP upregulated its expression in this anatomical segment. None of the rest of the treatments displayed any significant difference ($p > 0.05$).

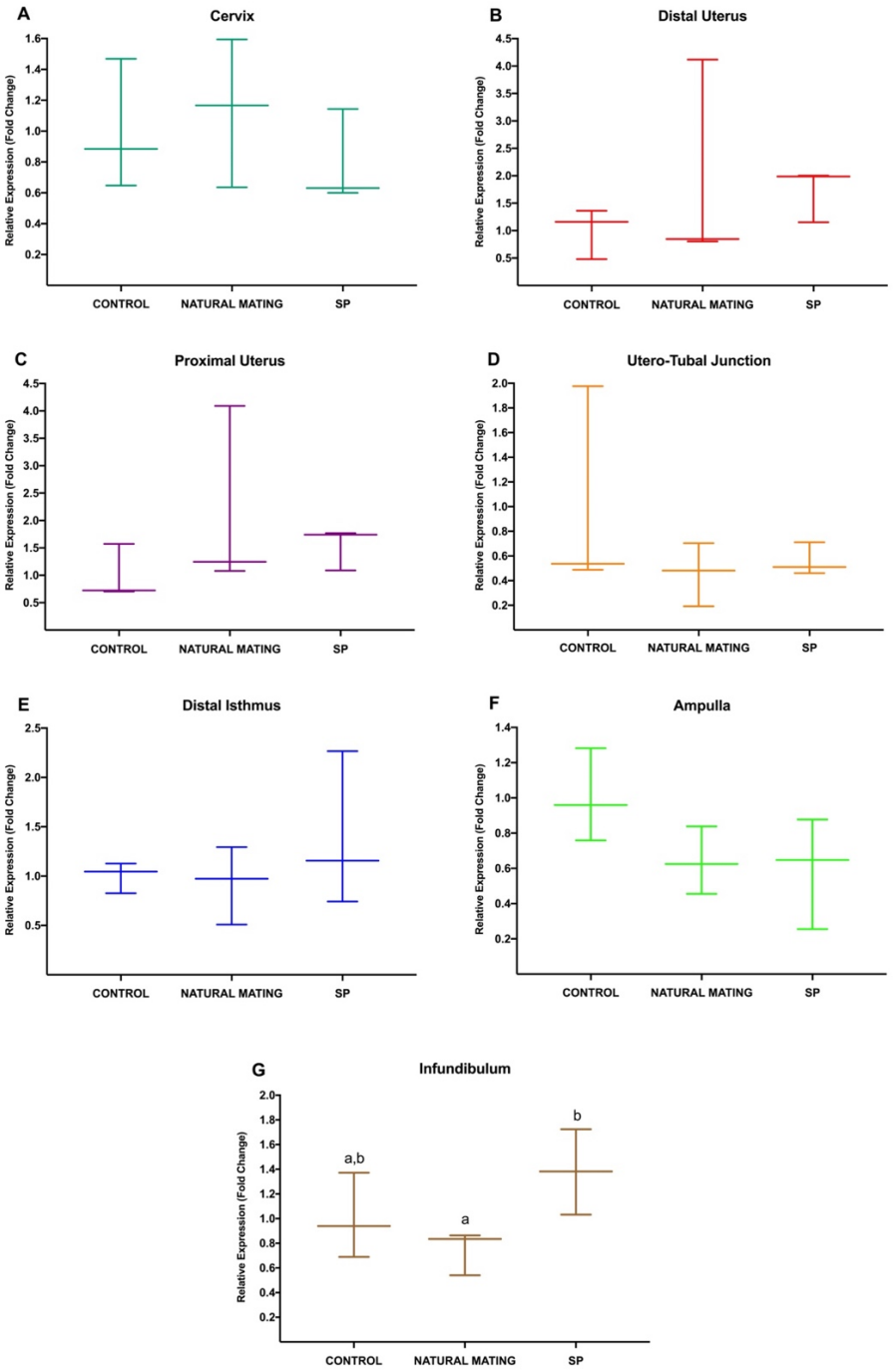

Figure 1. (A–G). Gene expression differences in *NR3C1* in the different anatomical segments (cervix to infundibulum; Experiment 1) (A–G), between negative control, natural mating, and seminal plasma (SP) treatments. Different letters indicate values that differed significantly between treatments in the same anatomical region ($p < 0.05$). The expression was relativized using the negative control as a reference. Median (minimum, maximum).

Second, the expression changes triggered by each treatment were compared between anatomical regions in the doe reproductive tract, represented in **Figure 2**. In the negative controls (**A**), significant *NR3C1* upregulation was reported in Amp and Inf, relative to the rest of the anatomical segments ($p < 0.05$; except Cvx, $p > 0.05$) and also a downregulation in the uterus (DistUt and ProxUt), relative to Cvx, Amp and Inf ($p < 0.05$). In the case of sperm-free SP infusion (**C**), significant upregulation of *NR3C1* expression was shown in Inf compared to the rest of the anatomical segments ($p < 0.05$). Moreover, upregulation in Inf compared to UTJ was also found ($p < 0.05$) in the natural mating group.

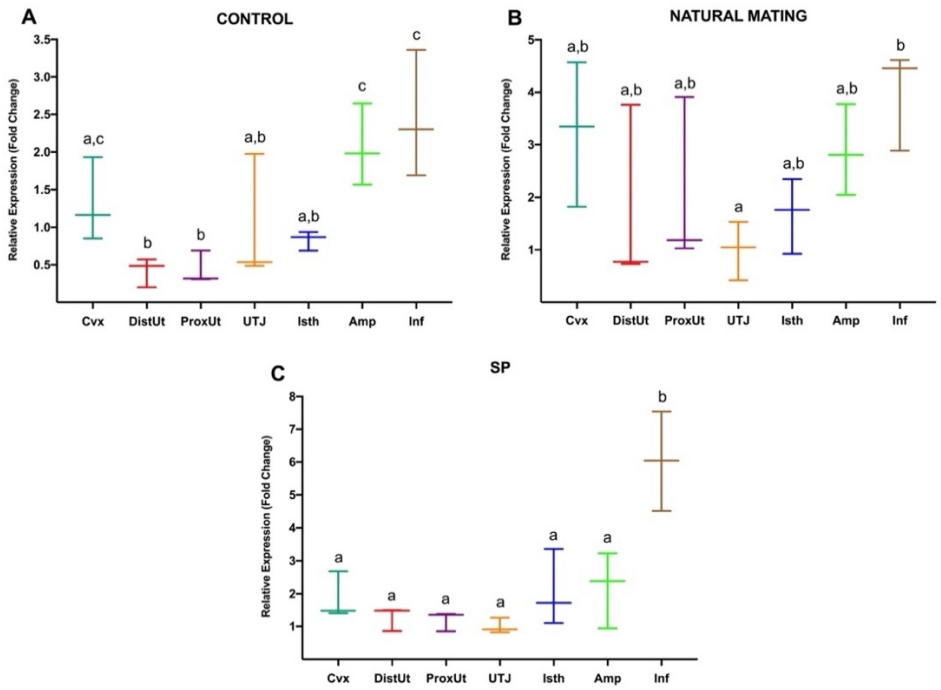

Figure 2. (**A**–**C**). Gene expression differences of *NR3C1* between anatomical segments (cervix, Cvx; distal uterus, DistUt; proximal uterus, ProxUt; utero-tubal junction, UTJ; distal isthmus, Isth; ampulla, Amp; infundibulum, Inf) in negative control group (**A**), natural mating (**B**), and sperm-free seminal plasma infusion (SP) (**C**). Different letters indicate values that differed significantly between anatomical regions in the same treatment ($p < 0.05$). The expression was relativized using the UTJ as a reference. Median (minimum, maximum).

EXPERIMENT 2: DIFFERENTIAL GENE EXPRESSION IN RABBIT FEMALE REPRODUCTIVE TRACT FROM 10 H TO UP TO 72 H IN RESPONSE TO NATURAL MATING

Figure 3 shows the results of *Experiment 2*, where 10 h post-mating was used to relativize the expression of the rest of the groups (24 h, 36 h, 68 h, 72 h post-mating). These timepoints are representative of embryo developmental stages. Thus, preovulatory stage, 2-4 cell embryo, 8-cell embryo, morula, and compacted morula correspond to 10, 24, 36, 68, and 72 h, respectively. In this sense, the number of ovarian follicles and embryos, and the present developmental stages, were evaluated and could be found in the aforementioned tissue collection description.

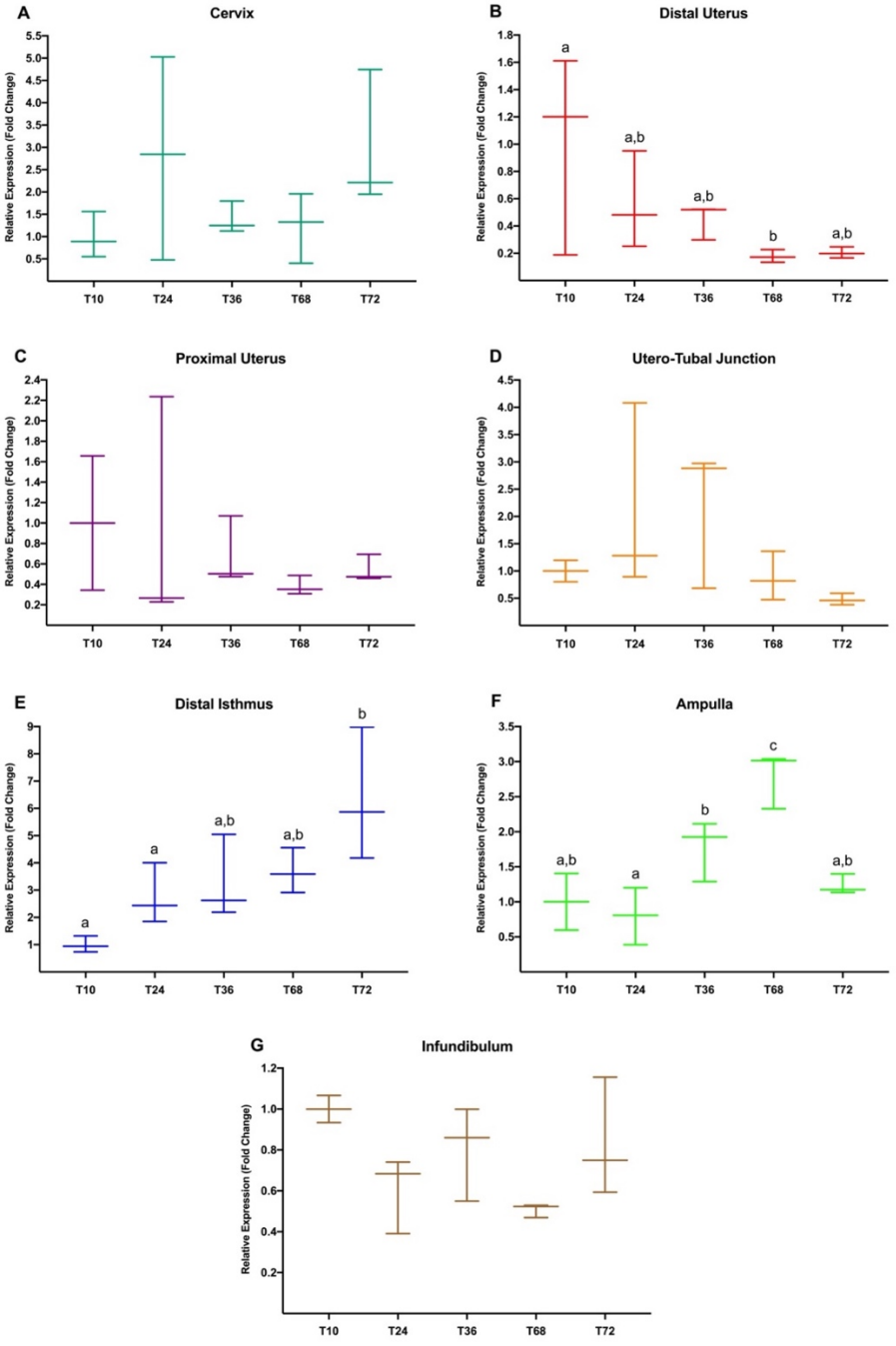

Figure 3. (A–G). *NR3C1* gene expression differences between each timepoint (T10, 10 h; T24, 24 h; T36, 36 h; T68, 68 h; and T72, 72 h; Experiment 2) post-natural mating in the different anatomical segments of the doe reproductive tract (cervix to infundibulum) (A–G). Different letters in the same anatomical region indicate values that differed significantly between timepoints ($p < 0.05$). The expression was relativized using the T10 as a reference. Median (minimum, maximum).

Significative differences in *NR3C1* expression were found in the DistUt (**B**), Isth (**E**), and Amp (**F**) at different times after mating. In the DistUt, a downregulation was found at 68 h when compared to *NR3C1* expression at 10 h post-mating ($p < 0.05$). Differently, in the Isth, *NR3C1* expression was upregulated, showing significant differences at 72 h ($p < 0.05$), when compared to 10 and 24 h post-mating. Moreover, in the Amp, the *NR3C1* gene was found to be significatively upregulated at 68h, when compared to the rest of the timepoints analyzed 10, 24, 36, and 72 h post-mating ($p < 0.05$). Upregulation at 36 h was also found when compared to 24 h in this anatomical segment ($p < 0.05$).

The expression changes triggered by mating in each timepoint were also compared among the different anatomical regions (**Figure 4**). At 10 h post-mating (**A**), *NR3C1* was upregulated in the Inf compared to the Isth ($p < 0.05$). At 36 h (**C**), the expression of this gene was upregulated in the Amp when compared to the DistUt, ProxUt, and UTJ ($p < 0.05$) and also upregulated in the Inf, compared to the DistUt ($p < 0.05$). Similarly, after 68 h post-mating (**D**), significative upregulation was found in the Amp compared to the rest of the anatomical regions ($p < 0.05$). Additionally, expression in the Cvx was upregulated compared to the DistUt and UTJ ($p < 0.05$), and *NR3C1* expression in the Isth was also upregulated when compared to the DistUt ($p < 0.05$). Finally, at 72 h post-mating (**E**), expression was higher in the oviduct (Isth, Amp, and Inf) and also the Cvx compared to the DistUt, ProxUt, and UTJ ($p < 0.05$). Additionally, the ProxUt was upregulated compared to the UTJ ($p < 0.05$).

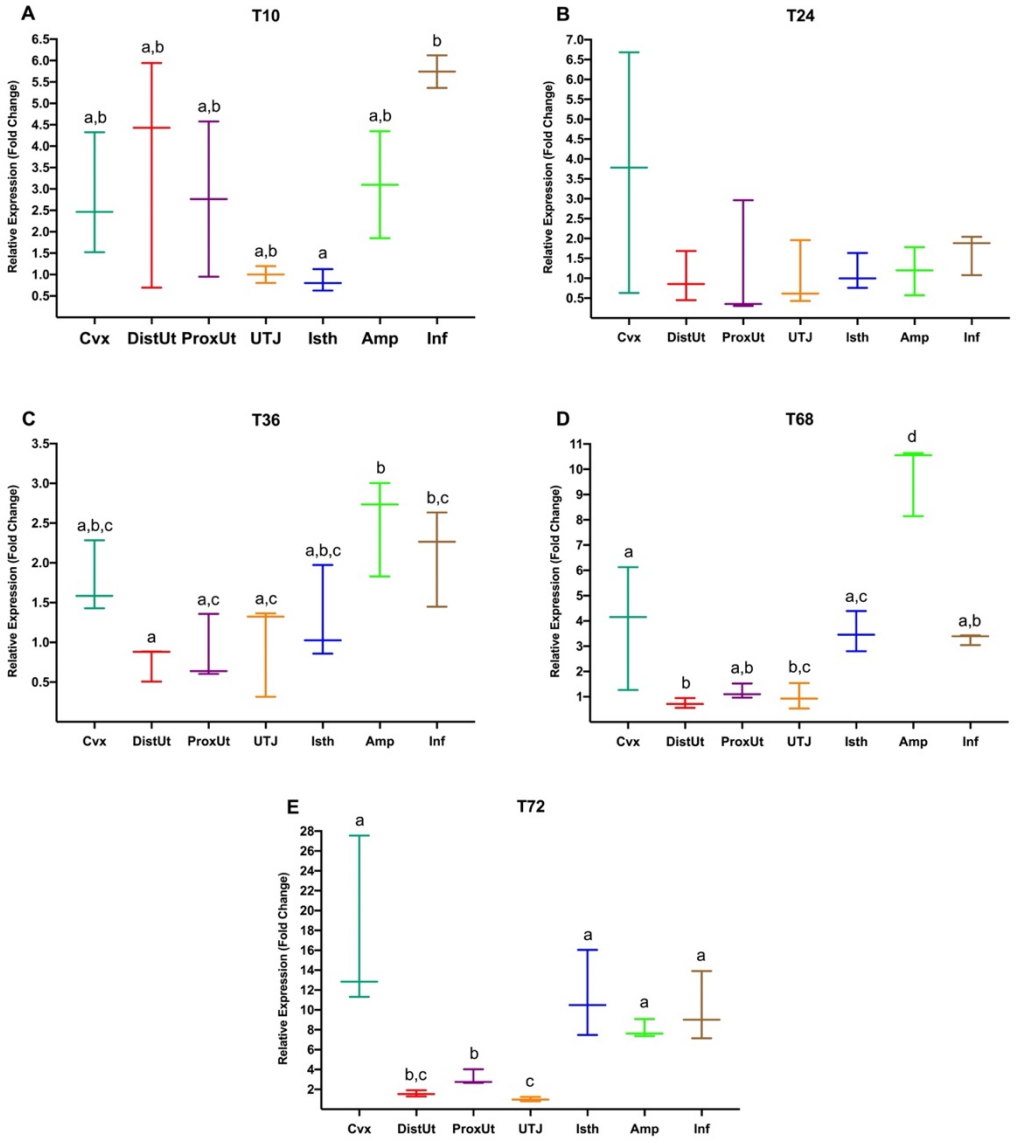

Figure 4. (**A**–**E**). *NR3C1* gene expression differences between anatomical segments (cervix, Cvx; distal uterus, DistUt; proximal uterus, ProxUt; utero-tubal junction, UTJ; distal isthmus, Isth; ampulla, Amp; infundibulum, Inf) in each of the timepoints 10 h (**A**, T10), 24 h (**B**, T24), 36 h (**C**, T36), 68 h (**D**, T68), and 72 h (**E**, T72) post-mating. Different letters in the same timepoint indicate values that differed significantly between anatomical regions ($p < 0.05$). The expression was relativized using the UTJ as a reference. Median [minimum, maximum].

DISCUSSION

In the present study, we evaluated changes in the post-ovulatory expression of the GC receptor gene (*NR3C1*) in the internal reproductive tract (cervix to infundibulum) of the female rabbit. *NR3C1* gene expression differences relative to insemination treatments and anatomical regions were analyzed 20 h after sperm-free SP infusion or natural mating, with the purpose of comparing the effects exerted by the whole semen or the sperm-free SP portion of the ejaculate (*Experiment 1*), and additionally, after mating at different timepoints (10 h, 24 h, 36 h, 68 h, and 72 h) (*Experiment 2*), during specific stages, i.e., ovulation, fertilization, and the interval of early embryo development to the morula stage achievement.

In our study, at 20 h after treatment, when ovulation may have already taken place 10 h ago (Dukelow & Williams, 1967), we found an upregulation of the *NR3C1* expression in the infundibulum, the oviductal segment where the follicular fluid and the mature oocytes are released shortly after ovulation (Talbot *et al.*, 1999). The promotion of the receptor expression in the infundibulum was observed in both natural mated and control ovulated females, but the *NR3C1* expression was significantly higher after sperm-free SP infusion, e.g., in the absence of spermatozoa. Moreover, the upregulation in this region was significantly higher than in any other of the reproductive tract segments sampled in the study. Hence, GC receptor expression in the infundibulum seems to be generally increased after ovulation, but SP, either provided by infusion or directly by mating, may promote *NR3C1* action at this location. In this sense, SP, which is not only related to the spermatozoa transport, but also to the modulation of the immune response exerted upon sperm- free SP contact in the female reproductive tract (Robertson, 2005), has been proven to have a positive effect on fertility in a

variety of species (Schjenken & Robertson, 2020), including rodents (Robertson, 2007), pigs (O'Leary *et al.*, 2004), and humans (Sharkey *et al.*, 2007), and may also play a role in the GC pathway in the reproductive tract (Ruiz-Conca *et al.*, 2020). SP is produced by sexual accessory glands together with secretions from the epididymis (Castellini *et al.*, 2012) and, in rabbits, it seems to play a role in spermatozoa protection, fertilization (Bezerra *et al.*, 2019) and also immune modulation during development (Schjenken & Robertson, 2014). After natural mating and sperm-free SP infusion (Parada-Bustamante *et al.*, 2016), the female reproductive tract undergoes a variety of modifications that influence the initiation of controlled inflammatory response, ovulation stimulation, changes in the transcription of genes related to reproductive stages (Schjenken & Robertson, 2020) and early embryonic development (Martinez *et al.*, 2019, 2020). Thus, even when the SP, deposited in the cervix, does not directly reach the upper regions of the oviduct such as the infundibulum, proteins of this fluid may be absorbed by the endometrium and achieve the ovary via lymphatic route or, alternatively, by activating signaling cascades (Waberski, 1997) that may modify the ovulation in rabbit (Maranesi *et al.*, 2018). In this way, SP molecules may modulate the overall genetic expression on different anatomical segments of the reproductive tract and, in light of our results, SP may also promote the action of GCs in the infundibulum, mediated by *NR3C1,* 20 h after GnRH injection. The specific localization of this expression change produced at this particular timepoint may be linked to the effects of SP components in the inflammatory response produced in the ovulation process. In this sense, previous studies have identified specific SP factors directly involved in the induced-ovulation triggering, such as the nerve growth factor (β-NGF). In rabbits, this mechanism of ovulation induction seems to be more complex compared to what is found in other species (Kershaw-Young *et al.*, 2012; Maranesi *et al.*, 2018; Ratto *et al.*, 2019; Silva *et al.*, 2020). In that sense,

previous studies found local effects of SP in the ovary that increased the number of hemorrhagic anovulatory follicles (Garcia-Garcia *et al.*, 2018; Silva *et al.*, 2011), suggesting an indirect action of SP on the luteinizing hormone (LH) receptors (Bomsel-helmreich *et al.*, 1989; Ratto *et al.*, 2019). The differences that we found between the SP and natural mating (also containing SP) treatments, where the expression in the infundibulum seemed higher than in the rest of the tissues, although not significant, are also intriguing. In this sense, those differences between both treatments could rely on the different physical stimulation that has been reported to play an important complementary role in the rabbit ovulation, that is not required in the camelids (Garcia-Garcia *et al.*, 2018). Thus, whether the specific effect on *NR3C1* expression we observed in the infundibulum is modulated by particular SP molecules and which those molecules remains are to be elucidated, but it might be plausible that β-NGF plays a role in the GC receptor promotion that is produced at the time of the ovulation in the infundibulum.

At that moment, shortly after the LH surge, ovulation starts and the rupture of mature ovarian follicles occurs (Duffy *et al.*, 2019). After oocytes' release, an inflammatory-like response, very similar to other inflammatory reactions, is produced at this location (Espey, 1980; Richards *et al.*, 2008), stimulated by a variety of changes in the reproductive tract including angiogenesis, vascular permeability, and exhaustive cellular differentiation, together with the production of mediators associated with inflammatory processes, such as steroids, prostaglandins, and cytokines, which may, in turn, be released to the infundibulum after the follicle rupture (Duffy *et al.*, 2019). The GC levels can be locally regulated within the reproductive tract to serve precise functions depending on the reproductive stage temporal context (Whirledge & Cidlowski, 2010), or the specific anatomical region (Gross & Cidlowski, 2008). Therefore, the GCs, steroid hormones well-known for their anti-inflammatory actions, are very present in the

oviduct and may contribute to the phase transition by promoting healing and repair after ovulation (Myers *et al.*, 2007). This may also be supported by the direct detection of ten times higher total cortisol levels in the follicular fluid after the LH surge (Harlow *et al.*, 1997), together with increased levels of 11β-hydroxysteroid dehydrogenases (11β-HSD1) during the luteal phase compared to the follicular phase (Thurston *et al.*, 2003, 2007), responsible for the conversion of cortisone (11-dehydrocorticosterone in rabbits) into active cortisol (corticosterone in rabbits), which provides direct and indirect evidence of the important presence of GCs in this region.

Moreover, we found that the expression of the GC receptor is also modulated over time, corresponding to the different preimplantation embryo developmental stages, e.g., preovulatory, 2-4 cells embryo, 8-cell embryo, morula, and compacted morula that we checked, as some of the oviductal and uterine anatomical segments show very disparate expression values during the hours that were tested here (10, 24, 36, 68, and 72 h), which were only evaluated after natural mating. Here, the uterus displayed low values compared to what was found in the oviduct at 36, 68, and 72 h post-coitum. At 68 h, these uterine values were also decreased compared to the values recorded in the periovulatory phase (around 10 h), which may imply a GC action in the uterus during the period prior to embryo implantation. In this context, the widely known action of steroid hormones modulates the uterus to prepare a suitable environment for successful embryo implantation (Gong *et al.*, 2008). In that regard, the necessary proinflammatory effects of estradiol in the uterus, including edema, increased vascularization, and promotion of the immune antibacterial activity, have previously been shown to be antagonized by the action of GCs in different mammalian species including rat, baboon, and sheep (Rhen *et al.*, 2003; Witorsch, 2016), and may also apply to induced ovulation species, as in the case of the

lagomorphs (Ratto *et al.*, 2019). GCs have also been demonstrated to assist difficult estrogen action by blocking cell differentiation, development and growth in the uterus, which may inhibit embryo attachment (Ryu *et al.*, 1999), trophoblast invasion, and implantation (Bitman & Cecil, 1967; Johnson & Dey, 1980; Rhen & Cidlowski, 2006). However, even when high levels of GCs may be potentially detrimental for the action of estrogens, presumably predominant in the uterus at this stage, *NR3C1* uterus knockout mouse models resulted in impaired implantation, decidualization, and pregnancy (Whirledge *et al.*, 2015), demonstrating that certain levels of GC regulation may be essential for an adequate uterine function.

Interestingly, an upregulation of GC action seems to be present along the rabbit oviduct at subsequent post-ovulatory timepoints according to our results. Thus, the *NR3C1* expression was found to be especially increased in the ampulla, and also in the infundibulum, at 36 h after mating and ovulation induction, when the early embryos are supposed to be transported through this oviductal region while performing the initial cell divisions (8-cell stage). At 68 h post-mating, when the embryo may be at the morula stage, the expression in the entire oviduct is also upregulated, with particularly high levels in the ampulla. This was also found at 72 h when the expression of the receptor was promoted in all the oviductal anatomical segments (distal isthmus, ampulla, and infundibulum) and also in the cervix. By this time, the morula stage is fully achieved and the embryos may have already reached the distal isthmus (Greenwald, 1961; Higgins & Kane, 2003). Thus, at 72 h, the levels of *NR3C1* in the distal isthmus were importantly high compared to the expression observed at the periovulatory stage (10 h after GnRH injection), and also to the expression at 24 h, shortly after fertilization is assumed to take place in upper oviductal regions (Cole & Cupps, 1969). This high value of *NR3C1* displayed in the distal isthmus may be explained by the previously

described action of steroid hormones in smooth muscle (Barton *et al.*, 2020). In this tissue, where the receptor is very present (Goodwin *et al.*, 2008; Rog-Zielinska *et al.*, 2013), GC action may cause a decrease in the prostaglandin action (Andersen, 2002). Thereon, one of the functions of prostaglandins is the preimplantation embryo retention in the oviduct for approximately 3 days (72 h) by stimulating the oviductal smooth muscle contractility (Blair & Beck, 1977; Lindblom *et al.*, 1980; Spilman & Harper, 1975). Moreover, RU486 (mifepristone), a glucocorticoid receptor antagonist (Peeters *et al.*, 2008), has been shown to increase the oviduct smooth muscle contractile frequency in rabbit (Xi *et al.*, 1996). Thus, the decrease in prostaglandins, favored by the action of steroid hormones, may be the cause of the isthmic sphincter relaxation that allows the morula embryos to enter into the uterus on its way down towards the implantation site (Wånggren *et al.*, 2008). In light of these results, GC action seems to be present along the oviduct in a distribution that may correspond to the assumed spatio-temporal location of the rabbit embryos regarding anatomical segments and timepoints, especially at the morula stage. After ovulation, the oocytes, together with follicular fluid, are released to the oviduct, where there is cross-talk between the gametes, the embryo, and the oviductal regions (Fernandez-Fuertes *et al.*, 2018). In this way, *in vitro* studies showed that cortisol production, mediated by 11β-HSD1, increased during maturation, and continued high during fertilization (Tetsuka & Tanakadate, 2019), and cortisol supplementation has improved blastocyst development rates in bovine (da Costa *et al.*, 2016), indicating that the GC activation may be part of the complex processes taken place during fertilization and early embryo development. In this sense, different results have been found regarding the effect of GCs in oocytes and early embryo development, as the influence of their presence seems to be species-specific (Gong *et al.*, 2017). To our knowledge, this is the first time that GC receptor levels

have been described in the rabbit reproductive tract. In other species, high levels of cortisol did not affect oocyte metabolism in equine (Scarlet *et al.*, 2017), while some harmful effects have been reported in pig (Gong *et al.*, 2017; Yang *et al.*, 1999), and different results have been shown in mice (Andersen, 2003; Gong *et al.*, 2017). The levels of GC receptor are crucial in the GC regulation, however, the regulation of these hormones is complex and comprises steps that are still not completely described, involving a great number of molecules, such as 11β-HSD (Gong *et al.*, 2017) and peptidyl-prolyl cis/trans isomerase FK506-binding proteins (i.e., FKBPs immunophilins) (Ratajczak *et al.*, 2015; Scammell *et al.*, 2001), among others. Thereby, the actions of GCs seem to variate among species and the importance of their regulation in reproduction is still far from being fully understood.

CONCLUSIONS

This is the first time that expression of *NR3C1*, the GC receptor gene, has been assessed in the internal reproductive tract of rabbits. Our results showed that, after sperm-free SP infusion, in the absence of spermatozoa, there is an increase in *NR3C1* expression in the infundibulum compared to natural mating. In the experiment over time, the differential expression of *NR3C1* was detected not only close to the site of ovulation and fertilization (ampulla and infundibulum), but also in the endometrium (distal uterus). The differential expressions are present over the interval during which early embryo development occurs, which may suggest a relevant role of the GC action, mediated by *NR3C1* on oviductal and uterine embryo transport. These results pave the way for further analysis that may elucidate the exact mechanism involved in the *NR3C1* action as well as its potential applications increasing the efficiency of the ARTs.

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SUPPLEMENTARY MATERIALS

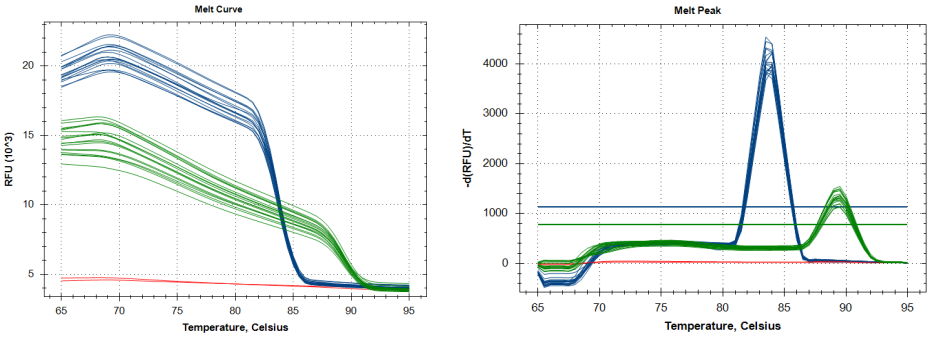

Figure S1. Melting temperatures of PCR products of *NR3C1* (blue) and *β-ACTIN* (green) in cervix tissue (36, 68, and 72 h post-mating groups) including negative template controls (red). For DNA-binding dyes (SYBR green), the fluorescence is brightest when the two strands of DNA anneal. Therefore, as the temperature rises towards the melting temperature (Tm), relative fluorescence units (RFU) decrease at a constant rate (constant slope). At the Tm there is a dramatic reduction in the fluorescence with a noticeable change in slope, displayed in the Melt Curve graph. The rate of this change is determined by plotting the negative first regression of fluorescence versus temperature (– d(RFU)/dT), displayed in the Melt Peak graph. The greatest rate of change in fluorescence results in visible peaks and represents the Tm of the double-stranded DNA complexes: 83.5 °C for *NR3C1*, and 89.5 °C for *β-ACTIN*.

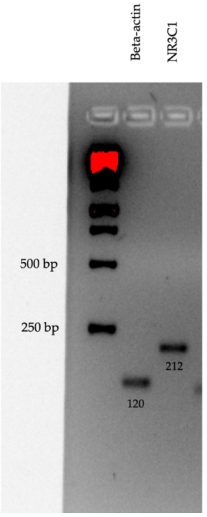

Figure S2. Agarose gel displaying PCR product size (bp: base pair) of *β-ACTIN* (120 bp) and *NR3C1* (212 bp).

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GENERAL DISCUSSION

Unsolved great challenges are ahead in the coming years regarding research in biology of reproduction, including alarming rates of biodiversity loss, sustainable development problems related to food supplies, and constantly declining fertility in humans linked to stress, unhealthy habits and pollutants (Barnosky *et al.*, 2011; Mascarenhas *et al.*, 2012; Palomba *et al.*, 2018; Segal & Giudice, 2019; Vollset *et al.*, 2020). The history of science is plenty of examples about how basic biology findings have been the cornerstone for the development of practical applications. It is a paradigmatic case the discovery of very particular genomic sequences of halophilic archaea (Mojica *et al.*, 1995). The technology derived from this discovery found in a small laboratory in Alicante reverted in the renowned CRISPR/cas9 genome editing tool (Lander, 2016), having countless applications for medicine nowadays. In this sense, both applied and basic research are essential for improving the assisted reproductive techniques (ARTs), such as cryopreservation (Hwang & Hochi, 2014), and identifying insufficiently described pathways involved in reproduction (Schimenti & Handel, 2018), including the glucocorticoid (GC) signaling (Whirledge & Cidlowski, 2017). In this thesis, both approaches are combined, contributing to the understanding of the GC regulation in the female reproductive tract physiology, and in the oocyte, where strategies towards the mitigation of the vitrification damage were also applied.

STRATEGIES TO MITIGATE OOCYTE DAMAGE

The oocyte is a very specialized cell that is formed during the early development of females. Primary oocytes remain arrested in prophase I at diplotene stage and the meiotic division is not completed until puberty is reached. Moreover, another arrest will occur in the oocyte after maturation, remaining in metaphase II (MII) until fertilization. Thus, some oocytes remain quiescent for a great number of

years or even decades, depending on the species. It is remarkable that a uterus can bridge three generations at the same time, since the primordial germline cells that will differentiate towards oocytes are developed during the female fetal development occurring in the uterus during pregnancy. During all these periods, the oocytes are exposed to aging and environmental insults, including stress, chemical endocrine disruptors, poor nutrition, an many other factors that can be pernicious (Andreas *et al.*, 2019; Van de Pette *et al.*, 2022). These detrimental influences may cause the increase of reactive oxygen species on the oocytes, inducing DNA damage, apoptosis and deleterious effects on the mitochondria (Moghadam *et al.*, 2022). Furthermore, in addition to the maternal nuclear genetic material, the oocyte provides a great contribution of cytoplasmic components to the future embryo, including mitochondria, mRNAs, non-coding RNAs, having an important influence on the embryo quality and developmental viability (Charney, 2013; Ruebel & Latham, 2020).

Hence, considering that the oocytes are sensitive cells, easily affected by external factors, it is then comprehensible that *ex vivo* oocyte management implies increased damage. Exposition to artificial *in vitro* maturation (IVM) conditions different than the maternal environment, including the routine use of non-defined media, or even to more challenging procedures such as cryopreservation can have a great impact on the oocyte (Agarwal *et al.*, 2005; Len *et al.*, 2019). In this context, there is a need of designing new strategies *in vitro* to overcome the detrimental impacts of the ARTs on the oocyte quality (Iwata, 2021; Roth, 2018). Thus, improving the reproductive outcomes, especially on species with particular conservation or commercial interest, could benefit from these approaches, as it is the case of the bovine (Hwang & Hochi, 2014). Accordingly, it was our aim to evaluate the application of coenzyme Q10 (Q10) supplementation

during IVM and vitrification of bovine oocytes to reduce, at least in part, the detrimental effects of this exogenous process.

COENZYME Q10 SUPPLEMENTATION OF IVM:

A USEFUL STRATEGY AGAINST OOCYTE DAMAGE DURING VITRIFICATION?

As a consequence of the low temperatures, the exposition to cryoprotectants, and the osmotic shock, vitrification is an stressful procedure for the oocytes (Arav *et al.*, 1996). Detrimental effects are induced to the oocytes during vitrification, causing damage to the meiotic spindle and the cell cytoplasm components (Hwang & Hochi, 2014), which correlates with our data of decreased rates of MII achievement, and increased premature exocytosis or incorrect migration of cortical granules

According to our findings, Q10 supplementation can be a real alternative to mitigate the detrimental effects of oocyte vitrification. At an appropriate concentration of Q10 (50 μM), increased rates of correct cortical granules migration were achieved after vitrification, forming a layer beneath the plasmatic membrane. These results imply a protective function of Q10 against a major pernicious effect of cryopreservation in the oocyte, the premature cortical granules release. Although the biological function of cortical granules is to block polyspermy, their premature exocytosis induces hardening of the zona pellucida, triggering a premature blocking of sperm fertilization (Rojas *et al.*, 2021; Wessel *et al.*, 2001). The Q10 is a lipophilic molecule that constitutes a component of the plasma membranes, being inserted between the phospholipids (Navas *et al.*, 2007). We hypothesized that Q10 supplementation during IVM may induce a stabilization of the oocyte plasmatic membrane by increasing its mechanical resistance to osmotic rupture. This advantage could be helping to overcome the

osmotic shock induced by cryoprotectants during vitrification. In this sense, detailed study of the cell membrane mechanical stability properties has pointed towards an increased Q10-mediated resistance to membrane rupture (Agmo Hernández *et al.*, 2015). In this sense, exposition to osmotic stress in *Escherichia coli* led to 110-fold incremented accumulation of coenzyme Q8 (Q8), the equivalent of Q10 on this species (Sévin & Sauer, 2014). Moreover, in the same study, Q8 demonstrated to be essential for acute and sustained osmotic stress tolerance in Q8 mutants. Supplementation of Q10 during IVM or *in vitro* culture has already shown beneficial effects in bovine (Gendelman & Roth, 2012; Stojkovic *et al.*, 1999) and also in pig (Yang *et al.*, 2021). Nonetheless, to our knowledge, this is the first time that Q10 has been applied as a strategy to overcome the damage caused by oocyte vitrification. However, the exact mechanism by which the Q10 is helping to preserve the oocyte integrity against vitrification is a question that may need further research, without discarding a combined action with many other factors.

Q10 has shown a wide range of different effects at cellular level, including protection from oxidative damage, changes in the mitochondrial function, apoptosis, and gene transcription (Crane, 2001; Quinzii *et al.*, 2010). In this sense, we investigated changes in the expression of bovine oocytes and cumulus cells, exposed to Q10 supplementation during IVM and vitrification. According to our findings regarding the decrease of pro-apoptotic expression genes and the increase of anti-apoptotic markers, Q10 supplementation during IVM may be exerting a protective effect against apoptosis of *in vitro* matured and vitrified oocytes. Similar effects of Q10 against stressful stimuli have been found on gene transcription in different cell types (Li *et al.*, 2019). While the underlying mechanisms responsible for this effect remains unclear, mitochondrial function may be playing a relevant role. The effects of Q10 on cells exposed to different

apoptotic stimuli showed that apoptosis prevention was independent of the free radical scavenger activity, and has been attributed to the prevention of mitochondrial membrane potential collapse (Papucci *et al.*, 2003). On the other hand, amelioration of the quality of aged oocytes, DNA damage and reduction of apoptosis has been linked to the mitigation of oxidative stress (Zhang *et al.*, 2019). Thereby, Q10 seems to have a beneficial effect against apoptosis in the oocyte after stressful events, and can be applied during reproductive procedures, such as IVM or vitrification.

GLUCOCORTICOID REGULATION IN THE FEMALE

The GC-mediated inhibition of reproduction during stress responses is a biological mechanism that is beneficial for the individual survival (Dhabhar, 2018). However, sustained stress levels may lead to reproductive dysfunction and infertility (Whirledge & Cidlowski, 2013). In the female reproduction, GCs does not have an straight inhibitory role, and some of its effects are actually essential for the normal function in the reproductive tract (Geraghty & Kaufer, 2015). In this sense, GC regulation is present during reproductive events, helping to achieve the *aurea mediocritas* (Aristotle, 1955). In other words, there are mechanisms for controlling the balance in GCs, *checkpoints* for separating between the necessity for appropriate function, and the pathogenicity of excessive exposure (Whirledge & Cidlowski, 2010; Whirledge & Cidlowski, 2017). Despite the relevance of this pathway, the regulation of GCs during reproductive events has not been unraveled, and the underlying mechanisms of action are complex and remain unclear (Geraghty & Kaufer, 2015). Moreover, differences between species may be present in the effect of GCs in the female reproduction (Gong *et al.*, 2017; González *et al.*, 2010a; Scarlet *et al.*, 2017; Yang *et al.*, 1999). The GC actions are

controlled by the GC availability, regulated by the enzymes HSD11B1 and HSD11B2 (Michael *et al.*, 2003), the participation of the GC receptor NR3C1 (Whirledge *et al.*, 2015), and the cofactors involved in the receptor translocation to the nucleus, including FKBP4 and FKBP5 immunophilins (Ratajczak *et al.*, 2015). How the expression of these factors is modulated in the oocyte and the female reproductive tract during the different reproductive events of animal species has remained *terra incognita* and it is one of the aims of this thesis to shed some light into this question.

GLUCOCORTICOID REGULATORY EXPRESSION IN THE OOCYTE

EFFECTS OF IVM, Q10 AND VITRIFICATION IN BOVINE

The role of GC in the oocyte and cumulus cells has remained a complex matter. After ovulation, a temporary local environment of GC is present during oocyte maturation (Tetsuka & Tanakadate, 2019), which is considered to be helpful for achieving the oocyte competence, at least in bovine (da Costa *et al.*, 2016; Santana *et al.*, 2014). Despite the described positive effects of GCs in the oocyte maturation of some species, an excess in GC production can have negative consequences in the oocyte (Andersen, 2003; Gong *et al.*, 2017; González *et al.*, 2010b; Yang *et al.*, 1999). Moreover, stress-induced levels of GC have important effects impairing the oocyte quality, inhibiting reproduction (Hong-Jie *et al.*, 2020; Prasad *et al.*, 2016; Zheng *et al.*, 2016). As mentioned before, the mechanisms by which GCs can damage or protect the oocytes are not currently well defined, but the mixed effects observed on each species has been attributed to the presence, absence, or proportions of the HSD11B1/B2 enzymes, and to the NR3C1 mediation (Gong *et al.*, 2017). In brief, to the mechanisms for the control of the GC availability and the GC receptor actions.

Understanding how GC actions are modulated may potentially open new options to improve the oocyte competence, or mitigate detrimental effects triggered by stress. For this purpose, we studied the modulation in the mRNA expression of genes involved in the GC regulatory activity occurring during the oocyte IVM. We are interested in how this GC regulation may change after oocyte vitrification, and supplementation with Q10, which seems a promising molecule for supplementation during IVM.

In this sense, *HSD11B1* and *HSD11B2* expressions are importantly regulated after Q10-supplemented IVM and vitrification of bovine oocytes and cumulus cells. According to our results, Q10 may be inducing a protective effect by increasing *HSD11B2* in cumulus cells after vitrification. This can be suggested based on the increased presence of the *HSD11B2*-encoded enzyme, responsible for inactivating cortisol into cortisone, in species showing greater resistance to GCs (Gong *et al.*, 2017; Scarlet *et al.*, 2017). Moreover, the inhibition of HSD11B2 decreases maturation rates of COCs in different species (Gong *et al.*, 2017). The effects of Q10 reducing *HSD11B1* may be also be positive for GC regulation, reducing an excessive exposure to GC that may be detrimental for the oocyte. In this way, an inhibition of HSD11B1 improved the maturation rates in pig oocytes, while had no effect in mouse oocytes (Gong *et al.*, 2017). However, progesterone increases the HSD11B1 activity during oocyte maturation in bovine (Anbo *et al.*, 2022), and the presumed effects of the Q10-induced changes in the expression should be interpreted carefully. In this respect, it is important to note that we did not test the activity of the enzymes, or checked the oocyte fertilization or embryo development rates.

We found evidences of *NR3C1* mRNA and protein expression in the bovine oocytes and cumulus cells, which changed after IVM, Q10

supplementation and vitrification. Despite being the receptor that regulates gene transcription and many more GC actions, the role of NR3C1 in the oocyte has remained unclear and there are surprisingly few evidences describing its presence in oocytes and cumulus cells (Čikoš *et al.*, 2019; Gong *et al.*, 2017). The oocytes seem to express more NR3C1 than embryos, and the lack of detection of NR3C1 in the oocytes some species, may be caused by the vast number of NR3C1 isoforms that are generated (Čikoš *et al.*, 2019). Here, we showed for the first time that NR3C1 expression in oocytes is increased after vitrification in presence of cortisol during bovine IVM, which might have implications regarding the oocyte resistance to GCs and the role of NR3C1 during oocyte maturation. However, the specific roles of NR3C1 isoforms and transcript variants in each species need to be determined by mechanistic studies and studied in detail.

We also found changes on the mRNA expression of *FKBP4* and *FKBP5* after IVM, which are implicated in the regulation of the NR3C1 translocation to the nucleus, and consequently, in the GC gene transcription actions (Ratajczak *et al.*, 2015; Wochnik *et al.*, 2005). While there are some reports of the importance of these immunophilins in the GC regulation in other reproductive cells (Hong *et al.*, 2007; Tranguch *et al.*, 2005; Yang *et al.*, 2006), to our knowledge this is the first description of their expression in oocytes and cumulus cells in any species. Their expression is increased during IVM, and their activity can constitute a relevant checkpoint in the regulation of the GC actions in the oocyte, regulating the NR3C1 transcriptional actions (Ratman *et al.*, 2013). Thereby, further studies focused on testing the effects of their functional inhibition in the oocyte or knockout models will add some light to their unexplored roles in this cell.

GLUCOCORTICOID REGULATORY EXPRESSION IN THE FEMALE REPRODUCTIVE TRACT

In mammals, the female reproductive tract is –in addition to spermatogenesis in the male– the environment where all reproductive events take place. The oocyte is released to the oviduct at ovulation during each estrous cycle and matured to reach metaphase II (Talbot *et al.*, 1999). Internal semen deposition, either by mating or the use of artificial insemination (AI), occurs inside the female, resulting in male-female interactions, that include gametes but also seminal plasma (SP) (Parada-Bustamante *et al.*, 2016; Pérez-Cerezales *et al.*, 2018; Robertson, 2005). From fertilization to parturition, all embryonic developmental steps are hold in the female reproductive tract, which displays defensive immune responses against pathogens, but also functional tolerance towards the hemi-allogeneic fetus and placenta. Every segment of the female reproductive tract is considerably specialized in their specific functions in the different reproductive events. In this view, it seems necessary to study the GC regulatory expression not only in the oocyte biology, but also in the female reproductive tract and the events that take place on it, including the estrous cycle, the male influence in the tract, and the early embryo development.

MODULATION ACROSS THE ESTROUS CYCLE IN BOVINE

Besides the GC regulation within the cumulus oocyte complex described in different species, it should be taken into account that the actual regulation occurring *in vivo* might not only be dependent on the activity of the oocyte and cumulus cells. All reproductive events from follicular development to parturition are held within the female reproductive tract, and GC regulation may also occur at tissue level during these events (Acosta *et al.*, 2005; Siemieniuch *et al.*, 2010;

Tetsuka, 2007). While the important regulation of sexual steroid hormones during the sexual cycle is much more described (Binelli *et al.*, 2018), there is a gap in the knowledge regarding the modulation of the GC actions during this period. Thereby, we aimed to describe the modulation across the bovine estrous cycle of the mRNA expression genes related to GC regulation occurring in different tissues of the female reproductive tract.

According to our results, there are great differences in this GC-related expression between the estrous stages. Interestingly, the spatial location of the changes matches with the temporal location of important reproductive events, i.e. in the ampulla during the post-ovulatory stage, and in the endometrium during the late luteal phase. These results support the hypothesis that GC regulation is important during reproductive events, including oocyte maturation.

The relative expression levels of *NR3C1*, *HSD11B1*, *HSD11B2*, *FKBP4* and *FKBP5* observed in the oviductal ampulla after ovulation suggest a contribution of this tissue to the GC regulation during oocyte maturation in bovine. We already discussed the evidences of the GC influence in the oocytes of different species, including the bovine, however the role that the oviductal HSD11B1/B2 expression, i.e. the GC availability, may have over the oocyte should be considered. In this sense, the post-ovulatory 3-fold higher expression of *HSD11B2* compared to *HSD11B1* that we found in the ampulla may suggest an oviductal HSD11B2-mediated control of the GC levels available to the oocyte *in vivo*. Nonetheless, these assumptions should be verified in detail by further studies checking the actual enzymatic activity, as the proportion between the transcripts of both enzymes may change after protein translation.

Moreover, the *NR3C1* higher expression found in the ampulla during this period indicates a relevant role during the post-ovulatory stage. The NR3C1

would bind to GCs and translocate to the nucleus to act as a transcription factor (Frank *et al.*, 2021; Wochnik *et al.*, 2005). While the extent of the GC actions and interactions in the nucleus is complex (Granner *et al.*, 2015; Petta *et al.*, 2016), it may contribute to the dynamic modifications occurring in the female reproductive tract during oocyte maturation and transport. In this sense, the *FKBP4* and *FKBP5*, which we found also higher expressed during this period, would be additionally controlling the GC actions in a ultra-short negative feedback loop regulation of the NR3C1 signaling (Yang *et al.*, 2006; Zannas *et al.*, 2016).

In the endometrium, the expression of steroid receptors different than NR3C1 was promoted during the late luteal phase, including estrogen and progesterone receptors, indicating a great regulation of these hormones during this period, when the progesterone levels are elevated and the estradiol levels are starting to raise. In this sense, it should be considered the promiscuity of the steroid receptors, which may bind to different steroid ligands (Baker *et al.*, 2015). While NR3C1 has a high affinity for GC, the mineralocorticoid receptor NR3C2, which was also higher expressed during the late luteal phase, shows high affinities for cortisol, corticosterone and progesterone. These aspects, together with the putative presence of receptor subtypes and isoforms, undeniably add complexity to the understanding of the steroid hormones signaling in the reproductive tract, and must be taken into consideration.

THE INFLUENCE OF MALE-FEMALE INTERACTION IN PIG

The female reproductive tract is extensively modulated by the male-female reproductive interactions, causing changes in mRNA and protein expression levels along the oviductal and uterine tissues. These changes involve immunological and transcriptional responses that can influence important reproductive functions,

such as sperm transport and storage, oviductal contractility, fertilization, embryo development and implantation (Almiñana *et al.*, 2014; Álvarez-Rodríguez *et al.*, 2020; Musavi *et al.*, 2018). We aimed to assess whether the male-female interactions, in terms of natural mating, sperm ejaculate AI or SP infusion can modulate the GC regulatory mRNA expression in the porcine reproductive tract.

To our knowledge, this is the first report of modulation of the GC-related expression in the female in response to the male influence in any species. According to our findings, the vast majority of the differentially expressed genes evaluated by us were triggered by natural mating, and not mimicked neither by AI nor SP infusion. Naturally, male-female reproductive interaction in mammals entails copulation, which does not take place during AI. Although ARTs are designed to replicate what occurs in nature, some of the physiological effects mechanically induced by natural mating may not be achieved during these procedures (Álvarez-Rodríguez *et al.*, 2020; Orihuela *et al.*, 2009; Parada-Bustamante *et al.*, 2016; Shafik *et al.*, 2006). Our data may raise questions regarding the influence of these specific gene differences on the reproductive success between natural mating and AI. Interestingly, our results suggest that natural mating triggers in the oviduct of pre/periovulatory sows the expression of a tight regulatory response towards a reduction of the GC availability (lower *HSD11B1* and higher *HSD11B2* expressions) and the control of the receptor-mediated functional activity in the nucleus (lower *FKBP4* and *FKBP5*).

Regarding the GC receptor, we found higher *NR3C1* expression in the isthmus after mating, which may relate to oviductal preparation for an scenario of increased GC levels in the oviduct after ovulation (Geraghty & Kaufer, 2015; Hillier & Tetsuka, 1998; Tetsuka, 2007). Although changes in the rest of the cofactors described above were not induced by SP, higher expression was also found in the

isthmus, suggesting a possible role of SP in the activation of the NR3C1 (Álvarez-Rodríguez *et al.*, 2020; Martinez *et al.*, 2019; Robertson, 2007; Schjenken & Robertson, 2020). In this regard, this complex fluid can trigger relevant transcriptional changes in the oviduct. As mentioned before, "controlled" GC actions are necessary for maintaining the corpus luteum and contributing to steroidogenesis, among other functions in the female reproductive tract (Rae *et al.*, 2004; Whirledge & Cidlowski, 2017; Andersen, 2002), and the NR3C1 receptor acts as an important transcription factor (Ratman *et al.*, 2013; Timmermans *et al.*, 2019; Weikum *et al.*, 2017). Thus, we described for the first time that a collective response towards regulating the GC levels and actions in the female pig reproductive tract is triggered after mating, suggesting that the detrimental effects of GC may be prevented in the sperm reservoir before ovulation (Mannowetz *et al.*, 2017; Miller *et al.*, 2016). These results should be interpreted carefully, since we map here a specific time, the pre-ovulatory phase, allowing only to have an instantaneous picture of a defined moment. Thus, further studies testing longer periods and timepoints are necessary to know whether this GC signaling is maintained over time.

MODULATION DURING EARLY REPRODUCTIVE EVENTS IN RABBIT

In induced ovulation species, such as the rabbit, the ovulation is triggered by mating, which is followed by high rates of fertilization and embryo development. Moreover, these events are presumed to occur at described specific ranges of time (Püschel *et al.*, 2010). These characteristics makes them of great interest for studying the early embryo developmental events (Fischer *et al.*, 2012). We aimed to evaluate the changes in the GC receptor mRNA expression, NR3C1, occurring in the different tissues of the rabbit female reproductive tract during early

reproductive events. To the best of our knowledge, this thesis is the first document describing the GC receptor levels in the doe reproductive tract.

We tested the effects of the SP, which induced higher expression of *NR3C1* in the oviductal infundibulum. Although anatomically far, the SP has already shown to induce relevant effects in the oviduct, and the importance of its actions should not be underestimated (Álvarez-Rodríguez *et al.*, 2020). The SP has roles in the immune regulation (Alvarez-Rodriguez *et al.*, 2019; Sharkey *et al.*, 2007), and its influence has been related to the factors triggering ovulation in induced-ovulator species (Adams *et al.*, 2016; Kershaw-Young *et al.*, 2012). Thus, NR3C1 may be having a role in this pathway and some of the specific functions of the NR3C1 in this regard deserve further considerations.

According to our results, the NR3C1 is modulated over time along the female rabbit reproductive tract after mating. Interestingly, our findings showed a pattern of higher expression of NR3C1 that corresponds with the assumed location of the rabbit embryos, at different preimplantation developmental stages. Increased levels were found in oviductal tissues at 36, 68 and 72 hours after mating, when the early embryos are presumed to be in 8-cell stage, morula and compacted morula stages, respectively, and are supposed to be transported throughout the oviduct. These results point towards a relevant function of the NR3C1 during the early embryo development, and consequently also the action of GCs, which may be exerting undescribed functions in this period. We found higher expression in the distal isthmus at 72 h, which may have a role in the isthmic sphincter contractility relaxation that allows the entrance of embryos to the uterus (Peeters *et al.*, 2008; Xi *et al.*, 1996). We encourage further research focused on the functions, of NR3C1 and co-regulators of the GC actions, in different species, which may help to better understand this complex pathway in reproduction.

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CONCLUSIONS

The chapters of this thesis include studies performed with the aim of widening the knowledge related to the role of the glucocorticoid regulation pathway in the reproductive function across animal species and the improvement of assisted reproduction techniques, by determining its modulation in the female reproductive tract during reproductive events, and during oocyte *in vitro* maturation, antioxidant supplementation, and vitrification.

The following conclusions can be drawn from this thesis:

Specific objective 1

To evaluate the effects of coenzyme Q10 supplementation during *in vitro* oocyte maturation as a potential molecule for attenuating the negative effects of vitrification.

Conclusion 1.1

Coenzyme Q10 supplementation (50 μM) during *in vitro* maturation prevents cortical granules exocytosis after vitrification of bovine oocytes, helping to preserve the oocyte integrity.

Specific objective 2

To examine the changes in glucocorticoid and apoptosis expression in bovine oocytes and cumulus cells after *in vitro* maturation, coenzyme Q10 supplementation and vitrification.

Conclusion 2.1

The glucocorticoid regulation pathway mRNA expression changes during bovine oocyte *in vitro* maturation and it is modulated by vitrification and coenzyme Q10 supplementation.

Conclusion 2.2

Coenzyme Q10 supplementation during bovine oocytes *in vitro* maturation decreases mRNA expression of apoptosis, especially after vitrification of oocytes and cumulus cells.

Conclusion 2.3

Coenzyme Q10 supplementation during *in vitro* maturation of bovine oocytes promotes the inactivation of glucocorticoids after vitrification, decreasing the expression of *HSD11B1* in oocytes and increasing the expression of *HSD11B2* in cumulus cells.

Conclusion 2.4

Cortisol supplementation (0.25 μM) during *in vitro* maturation of bovine oocytes increases the oocyte expression of the NR3C1 receptor protein after vitrification.

Specific objective 3

To determine the changes in glucocorticoid expression in the bovine endometrium and ampulla during different stages of the estrous cycle.

Conclusion 3.1

The glucocorticoid regulatory pathway mRNA and protein expression changes throughout the stages of the bovine estrous cycle.

Conclusion 3.2

The tight-regulation of the glucocorticoid-related expression is promoted in the ampulla during the post-ovulatory phase of the estrous cycle, including the increased mRNA expression of *NR3C1*, *FKBP4*, *FKBP5*, *HSD11B1* and *HSD11B2*, being potentially linked with the spatiotemporal location of oocyte transport and putative fertilization.

Conclusion 3.3

The glucocorticoid regulators and steroid receptors expression changes in the endometrium predominantly occur during the late luteal phase, some of them potentially linked to a regulation in the glucocorticoid availability in the uterus.

Specific objective 4

To assess the influence of natural mating, artificial insemination and seminal plasma infusion in the glucocorticoid mRNA expression in the reproductive tract of preovulatory sows.

Conclusion 4.1

Natural mating induces changes in the glucocorticoid related mRNA expression in the reproductive tract of the sow, that are different to those induced by artificial insemination and seminal plasma infusion.

Conclusion 4.2

Changes in the glucocorticoid related mRNA expression after natural mating show a decreased expression of *HSD11B1* and *FKBP4*, and an increased expression of *HSD11B2* and *FKBP5* in the oviduct, which seem to be linked to a restriction of the glucocorticoid effects in the sperm reservoir.

Conclusion 4.3

The STAT pathway mRNA expression is decreased after natural mating, revealing that this modulation could be potentially linked to the promotion of tolerance towards the spermatozoa in the female reproductive tract.

Specific objective 5

To evaluate the modulation over time of the glucocorticoid receptor mRNA expression in the rabbit female reproductive tract during early reproductive events after mating.

Conclusion 5.1

The glucocorticoid receptor *NR3C1* mRNA is differentially expressed throughout the tissues of the rabbit female reproductive tract.

Conclusion 5.2

The glucocorticoid receptor *NR3C1* mRNA expression is differentially increased in the oviductal infundibulum of the female rabbit reproductive tract after the infusion with sperm-free seminal plasma, compared to the effect of natural mating.

Conclusion 5.3

The glucocorticoid receptor *NR3C1* mRNA expression after mating is differentially modulated over time, showing higher expression along the oviduct, matching with the putative spatiotemporal locations attributed to the stages of the rabbit embryo development and transport throughout the female reproductive tract.