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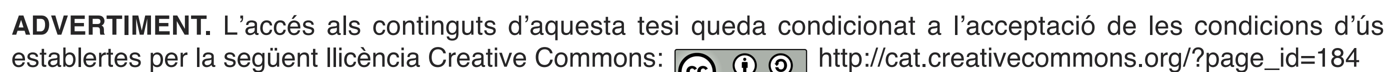
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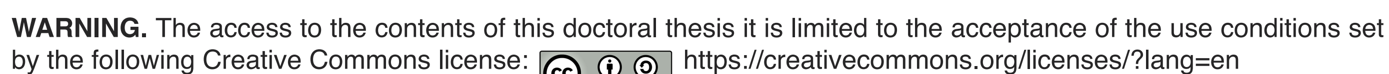
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COLD-INDUCIBLE RNA-BINDING PROTEIN: AN RNA CHAPERONE WITH POTENTIAL ROLES IN ANIMAL REPRODUCTIVE PHYSIOLOGY

DISSERTATION TO OBTAIN THE DEGREE OF DOCTOR
IN ANIMAL MEDICINE AND HEALTH BY:

Jaume Gardela Santacruz

UNDER THE SUPERVISION OF:

Dr. Manel López-Béjar

and

Dr. Manuel Álvarez-Rodríguez

PRESENTED TO THE DEPARTMENT OF ANIMAL HEALTH AND ANATOMY, FACULTY OF VETERINARY MEDICINE, UNIVERSITAT AUTÒNOMA DE BARCELONA

Bellaterra, March 2022

Manel López-Béjar, professor titular d'universitat del Departament de Sanitat i Anatomia Animals de la Universitat Autònoma de Barcelona, i

Manuel Álvarez-Rodríguez, investigador del Departament de Sanitat i Anatomia Animals de la Universitat Autònoma de Barcelona,

Certifiquen:

Que la memòria titulada "**Cold-inducible RNA-binding protein: an RNA chaperone with potential roles in animal reproductive physiology**", presentada per Jaume Gardela Santacruz amb la finalitat d'optar al grau de Doctor amb Menció Internacional en Medicina i Sanitat Animals, ha estat realitzada sota les seves direccions i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti a efectes oportuns, signen la present a Bellaterra, 3 de març de 2022.

Dr. Manel López-Béjar | Dr. Manuel Álvarez-Rodríguez

Jaume Gardela Santacruz
(doctorand)

Per a la realització de la tesi, l'autor va gaudir d'una beca pre-doctoral FI-DGR concedida per *l'Agència de Gestió d'Ajuts Universitaris i de Recerca* (AGAUR) de la *Generalitat de Catalunya* cofinançada amb el Fons Social Europeu (2018FI_B 00236).

Addicionalment, l'autor va gaudir de dues beques de mobilitat internacional concedides pel *Col·legi Oficial de Veterinaris de Barcelona* (Beques Ramon Turró) i pel *Servei d'Ocupabilitat de la UAB* amb finançament de la Comissió Europea (Erasmus+ KA-107 Mobility Fellowships between Program and Partner Countries).

Els diferents estudis inclosos a la present tesi doctoral han estat finançats pel *Ministerio de Ciencia e Innovación* (PID2019-108320RJ-I00, IJCI-2015-24380) i *Ministerio de Economía y Competitividad* (AGL2016-79802-P).

again and ever,

to you

"The path to your heart's desire is never overgrown."

– KIGEZI PROVERB, SOUTH-WEST UGANDA

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LIST OF ABBREVIATIONS AND ACRONYMS

A18 hnRNP	cold-inducible RNA-binding protein
Amp	ampulla
ANOVA	analysis of variance
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BTS	Beltsville thawing solution
cDNA	complementary DNA
CG	cortical granule
CIPs	cold-inducible proteins
CIRBP	cold-inducible RNA-binding protein
CIRP	cold-inducible RNA-binding protein
COC	cumulus-oocyte complex
CV	coefficient of variation
Cvx	cervix
DAMP	danger-associated molecular pattern
DC	detergent compatible
DEG	differential expressed gene
DistUt	distal uterus
DNA	desoxyribonucleic acid
E2	estradiol
EG	ethylene glycol
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FBS	fetal bovine serum
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
GRP7	glycine-rich RNA-binding protein 7
HPRT1	hypoxanthine phosphorribosyltransferase 1
IL-10	interleukin-10
IL-10RA	interleukin-10 receptor α subunit
IL-10RB	interleukin-10 receptor β subunit
IL-18	interleukin-18
IL-1β	interleukin-1β
Inf	infundibulum
INF	interferon

Isth	isthmus
IVM	*in vitro* maturation
JAK	Janus kinase
JTT	Jones-Taylor-Thorton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LME	linear mixed-effects
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NJ	Neighbor-Join
NLR	nucleotide-binding oligomerization domain-like receptor
NLRP3	NLR pyrin domain containing 3
P4	progesterone
PANTHER	protein analysis trough evolutionary relationships
PFA	paraformaldehyde
ProxUt	proximal uterus
PVDF	polyvinylidene difluoride
qPCR	quantitative real-time polymerase chain reaction
RBM3	RNA-binding protein motif 3
RBPs	RNA-binding proteins
RGG	arginine-glycine-rich
RIN	RNA integrity number
RIPA	radioimmunoprecipitation assay lysis buffer
RNA	ribonucleic acid
RNP	ribonucleoprotein domain
RRM	RNA recognition motif
SD	standard deviation
SEM	standard error of the mean
SP	seminal plasma
SRSF5	serine-arginine rich splicing factor 5
SRSFs	serine-arginine rich splicing factors
STAT	signal transducer and activator of transcription
TBP	TATA-box-binding protein
TRP	transient receptor potential
TRX	thioredoxin
UTJ	utero-tubal joint
WB	Western blot

SUMMARY

Vitrification has replaced the slow freezing method for cryopreservation of oocytes in several species. However, the optimal protocol for bovine oocyte cryopreservation remains to be established. Several strategies for improving bovine oocyte vitrification have been developed. In this regard, little attention has been given to sublethal mild hypothermia as an inductor of cryotolerance in bovine oocytes. The cold-inducible RNA-binding protein (CIRBP) belongs to the cold-inducible proteins (CIPs), a group of peptides induced by mild hypothermia and other stressors. CIRBP is a potential candidate to improve bovine oocyte cryotolerance due to its function in cell survival. In addition, CIPs play crucial roles in RNA transcript control, raising questions about the link between these proteins and the physiological changes in the female reproductive tract. Accordingly, the present thesis was conducted with the general objective to study CIRBP as a potential molecule to improve bovine oocyte vitrification and its modulation during physiological changes in the female reproductive tract. We first evaluated the effects of cold temperatures on the maturation of bovine cumulus-oocyte complexes. Additionally, we determined CIRBP protein expression by Western blot. Sublethal mild hypothermia was demonstrated to increase the expression of CIRBP. However, as expected, we reported detrimental effects induced by the sublethal stress. After this first approach, we aimed to find other strategies to increase the expression of CIRBP, avoiding the damaging effects of sub-physiological temperature variations. For this purpose, we tested different concentrations of exogenous CIRBP and a well-defined hypothermia mimetic small molecule (zr17-2), to supplement the *in vitro* maturation media of bovine cumulus-oocyte complexes. Minor changes in mRNA *CIRBP* expression and other CIPs were linked to this supplementation. Additionally, our results revealed that mild hypothermia and vitrification increased the mRNA expression of *CIRBP* and other CIPs. Finally, the female reproductive tract studies revealed that CIRBP is physiologically modulated by the cyclic hormonal changes and by the male-female interaction. Overall, this thesis offers new insight into CIRBP reproductive physiology, increasing the knowledge between CIRBP and the female reproductive tract and its potential use in improving bovine oocyte vitrification. A better understanding of the reproductive complex process may serve as novel clinical biomarkers, potential diagnostic tools for reproductive evaluation, and the improvement of animal production.

RESUMEN

La vitrificación ha reemplazado la congelación convencional para la criopreservación de ovocitos en varias especies. Sin embargo, aún no se ha establecido el protocolo óptimo para la criopreservación de ovocitos bovinos, aunque se han desarrollado varias estrategias para mejorar sus resultados. En este sentido, se ha prestado poca atención al estrés subletal por frío como inductor de criotolerancia en ovocitos bovinos. La *cold-inducible RNA-binding protein* (CIRBP) pertenece a las proteínas inducibles por frío (CIPs), un grupo de péptidos inducidos por hipotermia y otros factores de estrés. CIRBP es un candidato potencial para mejorar la criotolerancia de los ovocitos bovinos debido a su función en la supervivencia celular. Además, las CIPs desempeñan funciones cruciales en el control del ARNm, lo que plantea interrogantes sobre la relación entre estas proteínas y los cambios fisiológicos que se producen en el aparato reproductor femenino. En consecuencia, la presente tesis se llevó a cabo con el objetivo general de estudiar CIRBP como molécula potencial para mejorar la vitrificación de ovocitos bovinos y su modulación durante los cambios fisiológicos en el tracto reproductivo femenino. En primer lugar, evaluamos los efectos de la hipotermia en la maduración de los complejos cumulus-ovocito. Además, determinamos la expresión proteica de CIRBP mediante Western blot. Se demostró que el estrés por frío aumenta la expresión de CIRBP. Sin embargo, como se esperaba, se detectaron efectos perjudiciales inducidos por el frío. Tras esta primera aproximación, se buscaron nuevas estrategias para aumentar la expresión de CIRBP evitando los efectos perjudiciales del frío. Para ello, testeamos diferentes concentraciones de CIRBP exógena y una pequeña molécula hipotérmico-mimética ya establecida (zr17-2) como suplemento de los medios de maduración *in vitro*. Se encontraron pocos cambios en el ARNm de *CIRBP* y otras CIPs relacionados con esta suplementación, pero si se observaron incrementos de expresión del ARNm de *CIRBP* y otras CIPs debido a la hipotermia y vitrificación. Finalmente, los estudios en el tracto reproductivo femenino revelaron que CIRBP está modulada por los cambios hormonales fisiológicos y la interacción macho-hembra. Esta tesis ofrece una nueva visión de la fisiología reproductiva de CIRBP, tanto para el incremento de conocimiento básico como para su uso potencial en la mejora de la vitrificación de ovocitos bovinos. Una mejor comprensión del proceso reproductivo puede servir como nuevos biomarcadores, potenciales herramientas de evaluación reproductiva y mejora de la producción animal.

RESUM

La vitrificació ha reemplaçat la congelació convencional per a la criopreservació d'ovòcits en diverses espècies. No obstant això, encara no s'ha establert el protocol òptim per a la criopreservació d'ovòcits bovins, encara que s'han desenvolupat diverses estratègies per a millorar els seus resultats. En aquest sentit, s'ha prestat poca atenció a l'estrès subletal per fred com a inductor de criotolerància en ovòcits bovins. La *cold-inducible RNA-binding protein* (CIRBP) pertany a les proteïnes induïbles per fred (CIPs), un grup de pèptids induïts per hipotèrmia i altres factors d'estrès. CIRBP és un candidat potencial per a millorar la criotolerància dels ovòcits bovins a causa de la seva funció en la supervivència cel·lular. A més, les CIPs exerceixen funcions crucials en el control del ARNm, la qual cosa planteja interrogants sobre la relació entre aquestes proteïnes i els canvis fisiològics que es produeixen en l'aparell reproductor femení. En conseqüència, la present tesi es va dur a terme amb l'objectiu general d'estudiar CIRBP com a molècula potencial per a millorar la vitrificació d'ovòcits bovins i la seva modulació durant els canvis fisiològics en el tracte reproductiu femení. En primer lloc, es va avaluar els efectes de la hipotèrmia en la maduració dels complexos cumulus-ovòcit. A més, es va determinar l'expressió proteica de CIRBP mitjançant Western blot. Es va demostrar que l'estrès per fred augmenta l'expressió de CIRBP. No obstant això, com s'esperava, es van detectar efectes perjudicials induïts pel fred. Després d'aquesta primera aproximació, es van buscar noves estratègies per a augmentar l'expressió de CIRBP evitant els efectes perjudicials del fred. Per a això, es van testar diferents concentracions de CIRBP exògena i una petita molècula hipotèrmico-mimètica ja establerta (zr17-2) com a suplement dels mitjans de maduració *in vitro*. Es van trobar pocs canvis en el ARNm de *CIRBP* i altres CIPs relacionats amb aquesta suplementació, però si es van observar increments d'expressió del ARNm de *CIRBP* i altres CIPs a causa de la hipotèrmia i vitrificació. Finalment, els estudis en el tracte reproductiu femení van revelar que CIRBP està modulada pels canvis hormonals fisiològics i la interacció mascle-femella. Aquesta tesi ofereix una nova visió de la fisiologia reproductiva de CIRBP, tant per a l'increment de coneixement bàsic com per al seu ús potencial en la millora de la vitrificació d'ovòcits bovins. Una millor comprensió del procés reproductiu pot servir com a nous biomarcadors, potencials eines d'avaluació reproductiva i millora de la producció animal.

PREFACE

Before starting the main topic of this thesis, I would like you to introduce a brief etymological story of the term “chaperone”. The word comes from the French *chaperon* (originally from the Late Latin *cappa*, meaning “cape”), which refers to a hood. You may have in mind the well-known fairy tale about a young girl and a big bad wolf, *The Little Red Riding Hood* (French: *Le petit chaperon rouge*). However, I want you to remember a different meaning, attested in the Oxford English Dictionary in the 18th century.

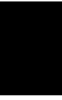
According to this definition, the term “chaperone” also means a person who accompanied an unmarried girl in public, especially if she was likely to encounter a man. Usually, this figure was conducted by an older married woman. The social term is obsolete and has fallen out of use in our time. However, this idea of “chaperon” or supervising a person was introduced in molecular biology to refer to proteins that participate in the folding or unfolding of conformational structures, as well as the assembly or disassembly of other macromolecular structures.

The term was coined by Ron Laskey and collaborators in 1978 to describe the ability of nucleoplasmin to avoid the aggregation of folded histone proteins with DNA during the formation of nucleosomes. R. John Ellis expanded the term in 1987 to include proteins that aid in the post-transcriptional assembly of protein complexes. Protein chaperones have been long associated mainly with protein folding, but a specialized group has RNA chaperone activity preventing RNA misfolding. The main character of this thesis, the cold-inducible RNA-binding protein, has RNA chaperone activity and is linked with low temperatures.

Ellis, J., 1987. Proteins as molecular chaperones. Nature 328, 378–379.

Laskey, R.A., Honda, B.M., Mills, A.D., Finch, J.T., 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature 275, 416–420.

INTRODUCTION



The most critical challenge of agriculture in the 21st century is to satisfy the food requirements of a globally growing population, primarily from developing countries, that will demand more production of crops and meat in the coming years (FAO 2017). However, properly feeding the world population would also imply adopting more efficient and sustainable production methods while adapting to climate change (Godfray *et al.* 2010; Agrimonti *et al.* 2021).

Cryoconservation[1] of germplasm[2] increases the efficiency of the livestock production systems, giving essential tools to breeders to continually adapt their animal genetic resources to the challenge of current changes on climatic and environmental conditions and the market economic demands (Morrell and Mayer 2017). Different cells have long been cryopreserved successfully, primarily due to the discovery and use of cryoprotectants, including semen (Polge *et al.* 1949), embryos, and oocytes (Mandawala *et al.* 2016). However, there are differences in the feasibility and practicality of these options. In this regard, oocytes are especially susceptible to slow freezing due to the low permeability to cryoprotectants of their plasma membrane and their large cytoplasm volume (Arav 2014). Nevertheless, other cryopreservation methods had been developed to preserve animal genetic resources germplasm. Among them, oocyte vitrification has displaced the slow freezing cryopreservation method (Mandawala *et al.* 2016). The key factor of the vitrification methodology lies in the absence of crystal formation during the solidification of the vitrification solution and the sample, minimizing the chilling injury of the oocyte membrane and organelles (Arav 2014). Although vitrification has been successfully used for the oocyte cryopreservation of several species, cattle are more problematic (Mandawala *et al.* 2016).

Different strategies have been implemented to cope with the low quality of bovine oocytes after vitrification (Mogas 2019), including modifying plasma membrane permeability, cytoplasm lipid contents, or even submitting the oocyte to sublethal stressors. In this line, sublethal stressors properly applied before manipulation could improve oocyte cryotolerance (Pribenszky *et al.* 2010). However, little attention has been given to mild hypothermia sublethal stress as a strategy to improve bovine oocyte vitrification.

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[1] The terms "cryoconservation" and "cryopreservation" are both used frequently throughout this and the following sections. Although these words are in some cases interchangeable, an effort has been made to restrict the use of "cryopreservation" to the actual process of freezing biological material for long-term storage. "Cryoconservation" refers to the conservation of animal genetic resources using cryopreserved germplasm.

[2] The term "germplasm" refers to all reproductive cells that, individually or in combination, are responsible for the reproductive performance and result in offspring development.

When exposed to mild hypothermia (34°C), mammalian cells decrease the overall protein abundance except for the expression of a small group of homologous glycine-rich mRNA-binding proteins known as cold-inducible proteins (Derry *et al.* 1995; Danno *et al.* 1997; Nishiyama *et al.* 1997; Tong *et al.* 2013; Rzechorzek *et al.* 2015; Fujita *et al.* 2017). Not only upregulated by mild hypothermia (Zhu *et al.* 2016), the cold-inducible proteins act as regulatory molecules with an essential role in RNA post-transcriptional control (Lunde *et al.* 2007). The cold-inducible RNA-binding protein (CIRBP), also called CIRP or A18 hnRNP, is one of the most studied members of the cold-inducible protein family (Zhong and Huang 2017). Its role in cell survival and anti-apoptotic cascades by binding to specific mRNAs (Zhu *et al.* 2016) makes CIRBP a potential candidate to improve bovine oocyte cryotolerance to vitrification.

However, reproduction is not only limited to the quality of the oocytes after vitrification and fertilization; the complex interactions between different physiological processes involved in domestic animal reproduction are also an interesting subject of study. Reproduction is a deep physiological process understood as an interplay of interactions between hormones, tissues of the female reproductive tract, male secretions, gametes, embryos, and the placenta (Wira *et al.* 2005). Even though both females and males play essential roles in reproduction, the female part deals with the highest portion of the reproductive processes, which start long before conception and last beyond birth (Estill 2015). Cyclic changes take part in the female mammalian reproductive tract to offer the optimal conditions for offspring development (Forde *et al.* 2011). Therefore, the female environment is in constant transformation and needs to adapt to the physiological requirements of every reproductive phase. In this regard, the role of the RNA-binding proteins in RNA post-transcriptional control raises questions about the actions of these proteins, including the cold-inducible proteins, during the physiological adaptations of the female reproductive tract. The importance of further understanding the complexity of the female physiological adaptations and studying the interplay between the female reproductive tract and the cold-inducible proteins is, therefore, evident.

Overall, this doctoral thesis is aimed to provide insights into the role of CIRBP in the regulation of bovine reproduction and how its modulation can benefit assisted reproduction techniques. **Chapter I** considers mild hypothermia as a sublethal stressor to increase CIRBP expression as a strategy to improve bovine oocyte vitrification. **Chapter II** presents the *CIRBP* mRNA changes in bovine oocytes and cumulus cells after being exposed to mild hypothermia and vitrification. **Chapter III** focuses on the mRNA modulation of *CIRBP* and other cold-inducible proteins during the bovine estrous cycle. And finally, **Chapter IV** presents the transcriptomic modulation of *CIRBP* and other RNA-binding proteins induced by the interaction between the female reproductive tract and male gametes and seminal plasma in swine.

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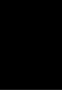
LITERATURE REVIEW



Conservation of animal genetic resources

Food and agriculture are essential for the livelihood of humanity, but livestock husbandry is in a period of change and upheaval (FAO 2017). Demand for animal goods is increasing due to general economic development and exponential population growth, but it has also put a strain on rural ecosystems and animal production systems to remain sustainable (FAO 2017). To fulfill this expanding demand, livestock breeders must raise their efficiency while adapting their animal genetic resources to changing economic and environmental situations (Godfray *et al.* 2010; Hoffmann 2010). The genetic diversity required for this adaptation is dwindling, and the genetic resources that remain are not being used to their full potential (Taberlet *et al.* 2008).

During the first *International Technical Conference on Animal Genetic Resources for Food and Agriculture* in Interlaken, Switzerland (September 2007), the Member Nations of Food and Agriculture Organization (FAO) developed the *Global Plan of Action for Animal Genetic Resources* (FAO 2007). This plan outlines four strategic priority areas for the long-term use, sustainability, development, and conservation of animal genetic resources worldwide. However, different reasons lead to conserving animal genetic resources. In developed countries, traditions and cultural values are important driving forces in conserving breeds at risk and the emergence of niche markets for livestock products (Taberlet *et al.* 2008; Sponenberg *et al.* 2018). On the other hand, in developing countries, the immediate concerns are food security and economic development (Godfray *et al.* 2010).

Conservation of animal genetic resources involves both *in vivo* maintenance and management of genetic diversity within livestock populations and *ex situ – in vitro* handling storage of genetic material that can be used later on to increase diversity in live populations or re-establish a population (Delgado Bermejo *et al.* 2019). The specific objective or objectives for conserving animal genetic resources will influence the strategy to conserve them. The strategies can be categorized as *in situ* conservation or *ex situ* conservation (Delgado Bermejo *et al.* 2019). Because both strategies differ in their capacity to achieve different objectives, *in situ* and *ex situ* conservation are complementary rather than mutually exclusive.

In general terms, the objectives for animal genetic resources conservation fall into the following categories (FAO 2012):

- Domestic animal diversity should be preserved for its economic value in helping the livestock industry to adjust to changes in agro-ecosystems, market needs and regulations, external input availability, disease issues, or a combination of these variables.
- The diversity of domestic animals performs a significant social and cultural role. As a result, the extinction of traditional breeds entails a loss of cultural identity for the community concerned, as well as a loss of a part of humanity's heritage.
- Domestic animal diversity is essential for the environment and the production systems. The loss of diversity in these production systems would increase instability and risk, as well as their inability to respond to change. Adapted breeds must be maintained and developed to ensure that food security may be reached sustainably without causing environmental harm.
- For research and training purposes: genetics, nutrition, reproduction, immunology, and adaptation to climatic and other environmental changes.

In situ conservation

In situ conservation refers to the preservation of animals in their natural habitats or production systems in which they were developed, ensuring the sustainable management of ecosystems used for agriculture and food production (Delgado Bermejo *et al.* 2019). This strategy also involves the active breeding of animal populations for food and agricultural production to ensure optimal diversity in a short time while simultaneously ensuring longer-term sustainability (FAO 2012). Activities about *in situ* conservation include performance recording schemes, development of breeding programs, and management of genetic diversity within populations (FAO 2012).

Ex situ conservation

Ex situ conservation means conservation away from the habitat and production systems where the resource developed (Mara *et al.* 2013; Delgado Bermejo *et al.* 2019). This category includes *ex situ – in vivo* conservation, in which germplasm is maintained in living animals, and *ex situ – in vitro* conservation, also known as cryopreservation.

Cryopreservation refers to collecting and deep-freezing of sperm, oocytes, embryos, or tissues for potential use in breeding or regenerating animals (Mara *et al.* 2013; Delgado Bermejo *et al.* 2019). This type of *ex situ* conservation enables the conservation of animal diversity away from the habitat and production systems where the source is developed. Additionally, wildlife can benefit

from these strategies using cryopreservation techniques optimized for livestock in their wild relatives or other non-related species (Holt and Pickard 1999).

Complementary roles of both strategies

In general, *in situ* conservation is the preferred strategy since it ensures that breeds are maintained in a dynamic state (Taberlet *et al.* 2008). Such idea may be accurate when the dynamics of a breed are characterized by slow and balanced adaptation to the conditions in which it is maintained. However, economically significant breeds are frequently subjected to intense selection pressures and higher-than-desired levels of inbreeding, whereas commercially less-important breeds have a small population size and are at risk of genetic drift and extinction (Taberlet *et al.* 2008). Moreover, conserving genetic diversity by keeping live animals outside their original or usual production environments will not always guarantee that the breed's genetic diversity is maintained (Taberlet *et al.* 2008). Thus, *in vivo* conservation should be complemented by cryoconservation of germplasm (De Oliveira Silva *et al.* 2021). In other words, establishing a germplasm bank may benefit long-term *in situ* conservation programs.

Germplasm management

As previously mentioned, all reproductive cells that, individually or in combination, are responsible for the reproductive performance and result in offspring development are referred to as “germplasm”. Therefore, cryopreservation of sperm, oocytes, and embryos is commonly used to ensure the widespread and long-term preservation of animal genetic resources (Mara *et al.* 2013; Morrell and Mayer 2017). Spermatogonial stem cells, testicular and ovarian tissue, and somatic cells for nuclear transfer are other sources of germplasm (Morrell and Mayer 2017).

Sperm

Because of its abundant availability and ease of application, sperm is one of the most practical ways to cryoconserve germplasm (Holt and Pickard 1999). Most mammalian species’ sperm has been successfully frozen since the cryoprotective effect of glycerol was unintentionally discovered by Polge *et al.* (1949) in the middle of the 20th century. However, the current semen cryopreservation protocols are species-specific, preventing the applicability across species from most mammalian species (Woods *et al.* 2004).

Oocytes

Due to the increasing use of oocytes in agricultural and scientific fields for procedures such as *in vitro* embryo production, nuclear transfer, and gene banking, oocyte cryoconservation is receiving more attention (Hwang and Hochi 2014). Like semen cryoconservation, oocyte cryoconservation benefits international germplasm exchange by lowering animal travel costs, eliminating animal welfare issues, and lowering disease transmission risks (Pereira and Marques 2008). Viable cryopreserved oocytes have long been recovered in several species, such as cattle, pigs, sheep, rabbits, mice, monkeys, and humans (Critser *et al.* 1997). However, oocytes suffer significant morphological and functional damage during cryopreservation that can be highly variable depending on the species, developmental stage, and origin (Mara *et al.* 2013). Despite many recent advances (Mogas 2019), the ideal bovine oocyte cryopreservation protocol has yet to be established.

Embryos

Successful embryo cryopreservation has been reported in more than 16 mammalian species since the first report of offspring using cryopreserved mouse embryos in 1972 (Whittingham *et al.* 1972). Embryo banking is an excellent alternative for conserving genetic diversity in species when collection and transfer processes are available and operational. Embryo cryopreservation is the fastest approach to reestablish an original breeding population (Marco-Jiménez *et al.* 2018), including nuclear and mitochondrial genetic information. On the other hand, it is usually more expensive and requires more technical expertise than semen gene banking (Dobrinsky 2002). Even though successful results have been reported obtaining live offspring from cryopreserved embryos in several livestock species, the expected success rate varies from species to species and the stage of the embryo, assuming especially good results with blastocysts (Dobrinsky 2002). Cattle present a high rate of success, in which embryo cryopreservation has become a routine procedure (van Wagtendonk-de Leeuw *et al.* 1997).

Cryopreservation principles and methods

Cryopreservation methods involve temperature reduction and cellular dehydration (Mazur 1963). Cryoprotectants are used in cryopreservation procedures to avoid ice formation and cold shock effects; however, they are linked with chemical toxicity and osmotic shock (Elliott *et al.* 2017). These compounds can be classified into permeating and non-permeating.

Permeating cryoprotectants (i.e., propylene glycol, ethylene glycol, glycerol, or dimethyl-sulfoxide) quickly enter cell membranes, form hydrogen bonds with intracellular water molecules, reduce the freezing point of the resulting mixture, and prevent intracellular ice crystallization (Elliott *et al.* 2017). Non-permeable cryoprotectants (i.e., sucrose, galactose, or trehalose), on the other hand, remain extracellularly, pulling free water from within the cell and dehydrating the intracellular space (Elliott *et al.* 2017). Both cryoprotectants are used in combination to increase the permeating cryoprotectant's net concentration inside the cell while simultaneously inhibiting ice crystal formation (Chen *et al.* 2005).

The two most used cryopreservation methods in animal reproduction are slow freezing and vitrification (FAO 2012), which are distinct approaches to the same physicochemical relationships.

Slow freezing

Slow freezing was the first cryopreservation technique developed (Whittingham 1971; Wilmut 1972; Willadsen *et al.* 1978). In this process, germplasm is exposed gradually to relatively low concentrations of permeable cryoprotectants or a combination of permeating and non-permeating (Chen *et al.* 2005). When germplasm is exposed to cryoprotectants, generally in small volumes into straws, the temperature is decreased between -5 and -7°C and maintained for several minutes to equilibrate (Saragusty and Arav 2011). After equilibration, the solution is seeded to induce extracellular crystallization and cooled down at about 0.3-0.5°C/min, reaching a final temperature between -30 and -65°C. Then, the straws are plunged and stored in liquid nitrogen (-196°C) once they have reached the final desired temperature. While temperature decreases during this procedure, extracellular ice masses containing pure crystalline water form (Mazur 1963). The solute concentration of the unfrozen fraction increases as the volume of this fraction decreases. The increase in osmotic strength drives an efflux of water from the cells. Slow cooling down is essential to allow enough water outflow to minimize the chance of intracellular ice formation. At the same time, the unfrozen fraction viscosity becomes too high to allow any further crystallization, which, in the end, becomes an amorphous solid with no intracellular ice crystal formation (Mazur 1963). Thereby, the intracellular matrix vitrifies.

Vitrification

Vitrification means transforming a liquid into a non-crystalline solid by lowering the temperature. Any process resulting in "glass formation" is vitrification. According to this definition, cells that are properly slow frozen are also "vitrified". But how does the so-called vitrification method differ from slow freezing if cells eventually become vitrified?

It would be incorrect and overly simplistic to define the difference between the two cryopreservation methods by saying that slow freezing uses slow cooling rates and low cryoprotectant concentrations, whereas vitrification uses fast cooling rates and high cryoprotectant concentrations. To achieve vitrification three important factors should be considered: cooling rate, the sample's viscosity, and sample volume (Saragusty and Arav 2011). The following general equation for the probability of vitrification is obtained by combining these three parameters:

$$Probability\ of\ vitrification = \frac{cooling\ rate \times viscosity}{volume}$$

To achieve ultra-rapid cooling rates, samples are directly plunged into liquid nitrogen, reaching cooling rates of thousands of degrees Celsius per minute. Several factors affect the cooling and warming rates achieved, such as the container, the sample volume, the thermal conductivity of the device used, and the composition of the vitrification solution (Yavin and Arav 2007). The sample's viscosity is defined by the concentration of cryoprotectants and other solutes of the vitrification solution (Saragusty and Arav 2011). The glass transition temperature rises with the concentration of cryoprotectants, lowering the risk of ice nucleation and crystallization. Smaller volumes provide better heat transfer, resulting in higher cooling rates. By reducing the sample volume and increasing the cooling rate, the concentration of cryoprotectants can be reduced moderately, reducing their detrimental toxic and osmotic effects (Yavin *et al.* 2008).

The vitrified state and the related physicochemical conditions resulting from vitrification and slow freezing are similar; however, the path to this point differs significantly. The lack of sophisticated equipment to manage the cooling rate makes vitrification a relatively cheaper method than slow freezing and a helpful method in field conditions, making it a good alternative for use in wildlife species and fieldwork in remote locations (Saragusty and Arav 2011).

Challenges of mammalian oocytes cryopreservation

In terms of cryopreservation, oocytes differ significantly from sperm and embryos. The following are some of the drawbacks of cryoconservation of mammalian oocytes (Figure **1**):

- The volume of a mammalian oocyte is three to four orders of magnitude greater than that of spermatozoa, resulting in a significantly lower surface-to-volume ratio, making them extremely sensitive to freezing and prone to intracellular ice formation (Toner *et al.* 1990; Ruffing *et al.* 1993; Arav *et al.* 1996; Zeron *et al.* 1999).
- The permeability coefficient of the plasma membrane of metaphase II oocytes is low, reducing the movement of cryoprotectants and water (Ruffing *et al.* 1993). Additionally, the glycoprotein layer surrounding the oocyte's plasma membrane, known as the zona pellucida, acts as an extra barrier to the movement of water and cryoprotectants into and out of the oocyte (Díez *et al.* 2012).
- Premature cortical granule exocytosis may occur due to the freeze-thawing process, resulting in zona pellucida hardening and making sperm penetration impossible (Mavrides and Morroll 2005; Coticchio *et al.* 2010). This process can be avoided by using additional assisted reproductive techniques such as the intracytoplasmic sperm injection (Coticchio *et al.* 2010).
- The high cytoplasmic lipid content present in oocytes increases chilling sensitivity (Ruffing *et al.* 1993). Moreover, their membrane is less robust because of the low presence of submembranous actin microtubules (Gook *et al.* 1993).
- Cryopreservation procedures cause cytoskeleton disorganization, and chromosome and DNA abnormalities (Díez *et al.* 2012). The meiotic spindle, formed by the metaphase II stage, is extremely sensitive to chilling and may be compromised as well (Gomes *et al.* 2012). However, it tends to recover to some level after thawing or warming, with vitrification-induced recovery faster than slow freezing-induced recovery.
- Oocytes are more vulnerable to the damaging effects of reactive oxygen species; therefore, the reactive oxygen species produced during the cryopreservation procedures might compromise the viability and developmental competence (Díez *et al.* 2012).

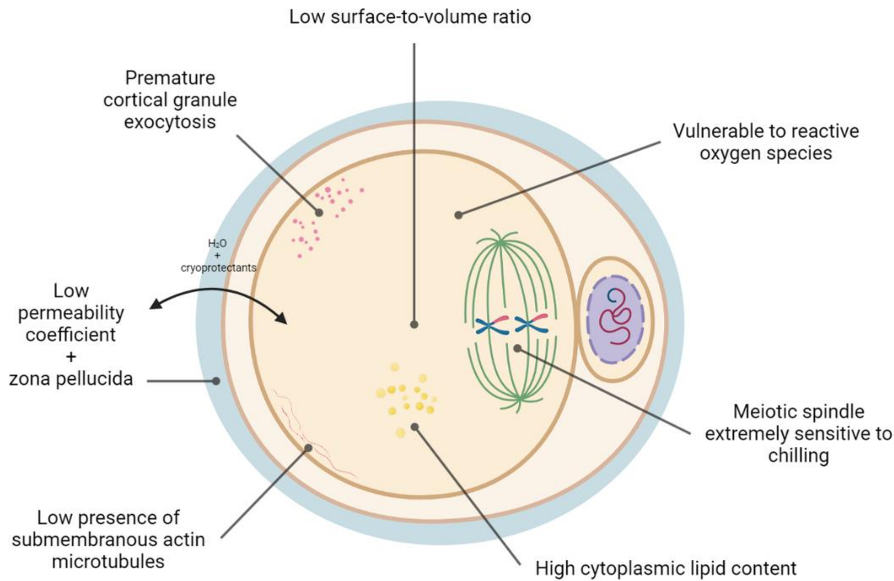


Figure 1. Main challenges during mammalian oocyte cryopreservation techniques. Oocytes are particularly sensitive to cryopreservation due to their low surface-to-volume ratio and susceptibility to reactive oxygen species. Additionally, the meiotic spindle formed in the metaphase II stage is highly susceptible to cooling effects. Their high cytoplasmic lipid content and their low presence of submembranous actin microtubules increase their sensitivity to cryopreservation procedures. Furthermore, the movement of water and cryoprotectants is restricted due to their low permeability coefficient and the zona pellucida. Finally, the cryopreservation procedures can induce premature cortical granule exocytosis, impairing fertilization.

Although the cryopreservation of oocytes is still considered experimental, especially for cattle (Mogas 2019), some advances and encouraging results have been obtained (Vajta *et al.* 1998; Dinnyés *et al.* 2000; Papis *et al.* 2000; Vieira *et al.* 2002; Chian *et al.* 2004). However, we should not forget that better results were reported using vitrification instead of slow freezing (Martínez-Burgos *et al.* 2011; Rienzi *et al.* 2016).

Strategies to improve bovine oocyte vitrification

Technical modifications to current bovine oocyte vitrification protocols are areas for future improvement (Díez *et al.* 2012). Potential strategies include adjustments on the vitrification protocol such as modifications of the cryoprotectant exposure regimes (García-Martínez *et al.* 2021) or changes in the cooling and warming rates using different vitrification devices (Saragusty and Arav 2011). However, other strategies are focused on manipulating the oocyte cellular composition to improve cryotolerance (Díez *et al.* 2012; Clark and Swain 2013; Mogas 2019), including, for example, modifications in plasma membrane permeability, cytoplasm lipid content, or submitting oocytes to sublethal stress before vitrification.

Stress for stress tolerance

Friederich Nietzche's opinion '*What does not kill me, makes me stronger*' (German: '*Was mich nicht umbringt, macht mich stärker*') outline the main idea behind this approach in his book published in 1889: *Twilight of the Idols, or, How One Philosophize with a Hammer* (German: *Götzen-Dämmerung, oder, Wie man mit dem Hammer philosophiert*).

Previous research suggests that sublethal stress, if properly defined and applied before manipulation, could improve oocyte cryotolerance by enhancing their eventual stress tolerance through adaptation mechanisms (Pribenszky *et al.* 2010). After various *in vitro* and *in vivo* processes, such as cryopreservation, parthenogenic activation, or somatic cell nuclear transfer, pretreatment with sublethal stressors appears to improve morphological intactness, fertilizing ability, and developmental competence of oocytes and embryos (Pribenszky *et al.* 2010; Pribenszky *et al.* 2012).

Osmotic stress and high hydrostatic pressure have been noted to improve both vitrification cryotolerance and developmental competence in gametes and embryos of several species (Pribenszky and Vajta 2011), while bovine oocytes seem to behave better exposed to high hydrostatic pressure (Muñoz *et al.* 2010) than osmotic stress (Arcarons *et al.* 2015). According to the theory (Pribenszky *et al.* 2010), these sublethal stressors lead to cell production and accumulation of chaperone proteins like the heat shock proteins. These proteins may help cells survive during the cryopreservation process, which is also a stress-inducing factor.

Cold shock and cold-inducible proteins

Despite the beneficial effects of sublethal stressors that have been attributed to the heat shock proteins (Pribenszky *et al.* 2010), little attention has been given to the cold-inducible proteins as potential candidates to increase the oocyte cryotolerance. In this regard, mild hypothermic temperatures and other cellular stressors, such as hypoxia, DNA damage, or osmotic stress, induce the expression of a group of RNA-binding proteins known as cold-inducible proteins (Zhu *et al.* 2016; Fujita *et al.* 2017).

The RNA-binding proteins assist in every step of RNA metabolism: transcription, splicing, polyadenylation, stabilization, edition, capping, and translation (Ciuzan *et al.* 2015). These proteins form ribonucleoprotein complexes by binding double or single-stranded RNA (Lunde *et al.* 2007). Their RNA-binding motifs or domains include, among others, the RNA recognition motif (RRM), K homology domain, double-stranded RNA-binding domain, or zinc fingers (Lunde *et al.* 2007). As RNA-binding proteins, the cold-inducible proteins participate in the regulation of RNA and are crucial for gene regulation (Zhu *et al.* 2016). These evolutionarily conserved RNA-binding proteins are found in several organisms from different taxa, both in prokaryotic and eukaryotic organisms (Ciuzan *et al.* 2015; Budkina *et al.* 2020).

Well studied in *Escherichia coli*, a sudden temperature downshift reduces colonial growth and almost a complete suppression of the overall protein synthesis (Jones *et al.* 1987). However, a group of proteins is highly expressed in response to cold shock (Jones *et al.* 1987; Etchegaray and Inouye 1999). Further research detected similar proteins in bacteria from different ecological groups involved in bacterial adaptation to low-temperature stress (Graumann and Marahiel 1998). Therefore, this group of proteins was named cold shock proteins or cold-inducible proteins. The presence of cold shock domains is a structural characteristic of the cold-inducible proteins (Budkina *et al.* 2020). These domains are represented by a β-barrel consisting of five antiparallel β-strands (Schindelin *et al.* 1994). The ribonucleoprotein domains (RNPs) RNP1 and RNP2, which are found in all the cold-inducible proteins, are found on strands β2 and β3 (Horn *et al.* 2007). The ability of prokaryotic cold-inducible proteins to bind nucleic acids is linked to all their known functions, regulating the synthesis of cell proteins by a variety of mechanisms, both at low temperatures and under optimal conditions for bacterial growth (Budkina *et al.* 2020).

In eukaryotic organisms, the cold-inducible proteins are composed of the cold shock domain and other domains, whereas prokaryotic cold-inducible proteins are nearly always composed of one cold shock domain only (Budkina *et al.* 2020). The eukaryotic cold shock domains have a spatial structure that is nearly identical to that of the prokaryotic ones (Skabkin *et al.* 2004), with five β-strands constructing the β-barrel (Schindelin *et al.* 1994). The primary distinction between

eukaryotic and prokaryotic proteins is that the former includes a longer linker sequence connecting the β3 and β4 strands and a different amino acid composition (Skabkin *et al.* 2004). The presence of additional domains in eukaryotic cold-inducible proteins allows them to expand their multifunctionality (Varadi *et al.* 2015) and act as organizers of various functional complexes, like the stress granules (Kedersha and Anderson 2007).

Cold-inducible RNA-binding protein

The cold-inducible RNA-binding protein (CIRBP, also known as CIRP, or heterogeneous nuclear ribonucleoprotein A18, hnRNP A18) is a stress-responsive 18-kDa protein that was first discovered in mouse cells in the late 1990s as a new RNA-binding protein whose expression was elevated in response to cold shock (Nishiyama *et al.* 1997). Investigations have revealed its significance in various cellular stress responses, including mRNA stability, cell survival, proliferation, stress adaptation, and tumor formation (Zhu *et al.* 2016; Liao *et al.* 2017; Zhong and Huang 2017; Aziz *et al.* 2019). CIRBP acts as an RNA chaperone regulating mRNA stability and translation intracellularly, but it also appears to have a role in inducing inflammation and injury outside of cells probably through the lysosome pathway (Qiang *et al.* 2013; Rajayer *et al.* 2013; Zhong and Huang 2017; Aziz *et al.* 2019). However, the exact mechanism by which CIRBP enters the lysosomal compartment remains unknown. Because CIRBP performs fundamentally different roles depending on its location, it is vital to distinguish between intracellular and extracellular CIRBP distinct traits and activities (Aziz *et al.* 2019).

CIRBP is structurally similar to the heterogeneous nuclear ribonucleoproteins and belongs to the glycine-rich RNA-binding protein family class IVa (Ciuzan *et al.* 2015). This class IVa glycine-rich protein subfamily's evolution is remarkably preserved in vertebrates and higher plants (Figure 2) in terms of primary amino acid sequences and their protein functions (Ciuzan *et al.* 2015). At the N-terminal end, CIRBP has one conserved RRM including two highly conserved RNPs sequences (RNP1 and RNP2), which has been shown to play a role in the posttranscriptional regulation of gene expression (Burd and Dreyfuss 1994). At the C-terminal end, a less conserved arginine-glycine-rich (RGG) domain is present, which could control CIRBP translocation in the cytoplasm and promote protein-protein interactions (Zhu *et al.* 2016; Zhong and Huang 2017). In response to cellular stress, CIRBP targets the translation of specific RNA transcripts with its RNA signature motif (Zhong and Huang 2017).

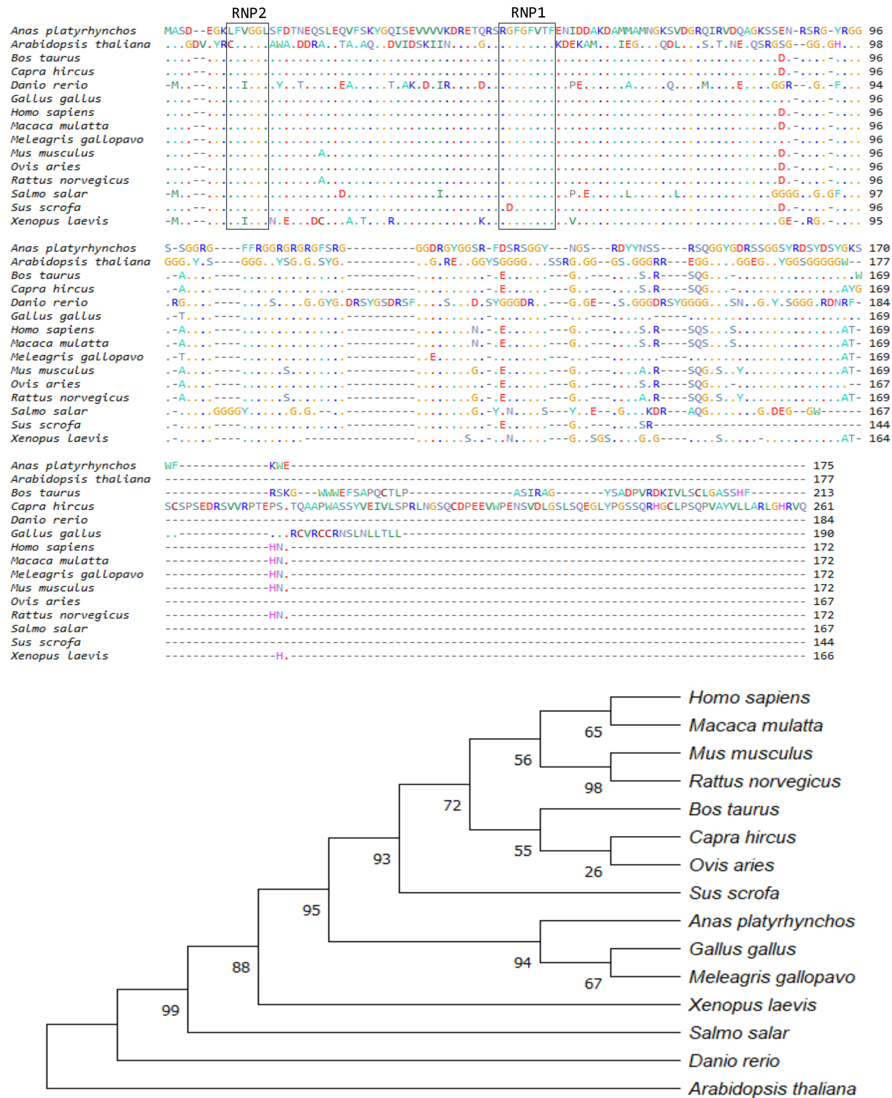


Figure 2. Protein alignment and homology tree of cold-inducible RNA-binding protein and its plant homologous (glycine-rich RNA-binding protein 7 – GRP7) in different species. Dots indicate amino acids identical between sequences. The sequence of the two ribonucleoprotein domains (RNP1 and RNP2) are marked in the alignment. The evolutionary history was inferred using the Maximum Likelihood method and the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.* 1992). The tree with the highest log likelihood (-1107.39) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 15 amino acids sequences. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021).

CIRBP is found in a wide range of cells, tissues, and organs from different species, including the brain (Tong *et al.* 2013), lungs (Hozumi *et al.* 2021), heart (Sano *et al.* 2015), kidneys (Peng *et al.* 2000), liver (Oishi *et al.* 2013), retina (Larrayoz *et al.* 2016), gonads (Gardela *et al.* 2019), and endometrium (Hamid *et al.* 2003). Its expression is upregulated in response to mild hypothermia, but also upon UV radiation, mild hypoxia, and glucose deprivation, suggesting that CIRBP is a general stress protein (Liao *et al.* 2017; Zhong and Huang 2017).

Multiple levels of regulation of CIRBP expression in response to stress are coordinated independently or collectively, including transcriptional activation, alternative splicing, and splicing efficiency (Gotic *et al.* 2016; Zhong and Huang 2017). Additionally, recent studies revealed the involvement of transient receptor potential channel proteins (TRPV3, TRPV4, and TRPM8) as potential regulators of CIRBP (Fujita *et al.* 2017; Fujita *et al.* 2018).

Hypothermia can be mimicked by small molecules

Predicting the binding activity with the flexible part of CIRBP and the RNA-binding site, Coderch and collaborators tested the capacity of a group of 15 selected small molecules to modulate CIRBP activity (Coderch *et al.* 2017). After the toxic *in vitro* assays, two compounds, zr17-2 (Figure **3**) and SD4, significantly elevated CIRBP protein expression in cultured cells at normothermia (37°C), with zr17-2 being more effective than hypothermia (32°C). *In vivo* assays demonstrated that zr17-2 increases CIRBP expression in a tissue-dependent way. No change in CIRBP expression was found in areas of the central nervous system and retina, whereas the peripheral tissues (lung, testis, heart, colon, pancreas, and subcutaneous fat) responded to zr17-2, increasing CIRBP expression levels (Coderch *et al.* 2017).

Figure 3. Chemical structure and molecular weight (g/mol) of zr17-2.

In the different binding models tested for zr17-2, the ligand was always found within the N- and C- terminal flexible ends, interacting with equivalent or closely related residues (Coderch *et al.* 2017). Identifying hypothermia mimetics that bind and modulate CIRBP expression at normothermia opens new approaches to use them in assisted reproductive techniques, such as oocyte vitrification.

Intracellular CIRBP

CIRBP actions modulate the expression of several genes; however, the localization of CIRBP determines its functions and effects on cells (Aziz *et al.* 2019). Primarily located in the nucleus, CIRBP controls RNA transcription and processing, while in the cytoplasm, CIRBP regulates mRNA translation and turnover (Nishiyama *et al.* 1997; Zhong and Huang 2017). The methylation of arginine residues in the RGG domain and phosphorylation at the C-terminal region regulate CIRBP translocation from the nucleus (De Leeuw *et al.* 2007; Masuda *et al.* 2012).

In the cytoplasm, CIRBP coordinates translational reprogramming by stimulating the synthesis of proteins (Aziz *et al.* 2019). Intracellular CIRBP bind to the 3'-untranslated region of ribosomal fraction RNA transcripts that contain the RNA signature motif of CIRBP (Zhong and Huang 2017). By attaching to its target mRNAs, intracellular CIRBP suppresses their deadenylation and retains their poly-A tail length, improving their stability and inhibiting their degradation (Aoki *et al.* 2003; Yang *et al.* 2010; Xia *et al.* 2013). All together lead to enhanced translational activity (Aoki *et al.* 2003; Yang *et al.* 2010; Xia *et al.* 2013). Indeed, intracellular CIRBP has been demonstrated to enhance the translation of genes involved in DNA repair (Haley *et al.* 2009; Yang *et al.* 2010), cellular redox metabolism (Yang *et al.* 2006), cellular adhesion (Peng *et al.* 2006), circadian homeostasis (Morf *et al.* 2012), reproduction (Xia *et al.* 2013), telomerase maintenance (Zhang *et al.* 2016), and translational machinery (Zhong and Huang 2017).

In addition to the positive posttranscriptional regulation, nuclear CIRBP migrates to cytoplasmic stress granules during stress conditions, where it acts as a translational repressor, negatively regulating protein synthesis in general (De Leeuw *et al.* 2007). This translocation is accompanied by binding to a subset of mRNAs that code for various stress-related molecules (Liao *et al.* 2017). These stress granules provide "shelter" for transcriptionally silent mRNAs in response to stress in cells (De Leeuw *et al.* 2007). When environmental stresses are no longer present, the stress granules disintegrate in dynein and microtubule-dependent manner, releasing the wrapped mRNAs for translation, allowing cells to recover quickly (Mokas *et al.* 2009).

The genes induced by intracellular CIRBP are assumed to be a function of the intensity (mild, moderate, or severe) and timing of the stressors (early or late exposure) (Aziz *et al.* 2019). During the initial stages of the stress response, intracellular CIRBP expression increases (Liao *et al.* 2017). Then, CIRBP translocates from the nucleus to assist the translation of several mRNAs, activating cell survival cascades (Figure 4) such as the NF-κB and ERK1/2/MAPK pathways, and thioredoxin (TRX)-mediated antioxidant process (Liao *et al.* 2017, Aziz *et al.* 2019; Bolourani *et al.* 2021). As the stress persists, toxic substances, like reactive oxygen species and abnormal proteins cumulate in the cytoplasm (Liao *et al.* 2017).

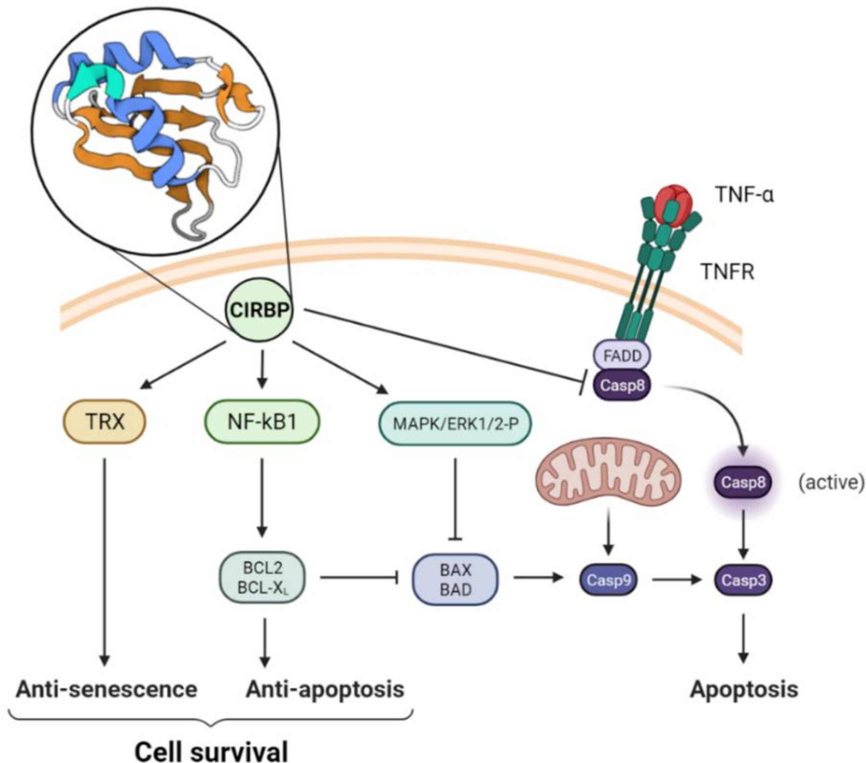


Figure 4. Under stress, CIRBP is involved in a cell survival cascade. Increases in CIRBP expression in response to stress help cell survival in the following way. Generally, stressors suppress global mRNA transcription and translation but activate CIRBP in most cases. CIRBP acts as a core death signal suppressor in the TNF-α signaling pathway, preventing caspase-8 activation. Meanwhile, CIRBP activates the MAPK/ERK1/2 and NF-κB pathways, reducing BAX and BAD action on caspase-9 and activating BCL2 and BCL-X_L anti-apoptosis cascades. In addition, CIRBP is hypothesized to suppress the senescence process by increasing TRX expression to reduce stress-induced reactive oxygen species. Modified from Liao *et al.* 2017.

Extracellular CIRBP

During hypoxia and inflammation, CIRBP is translocated from the nucleus to the cytoplasm and eventually released into the extracellular space (Qiang *et al.* 2013). Extracellular CIRBP has been proposed as a damage-associated molecular pattern (DAMP) in ischemic and inflammatory conditions (Aziz *et al.* 2019). In such disorders, CIRBP tissue and serum levels have been found to be elevated, as well as in chronic inflammatory states (Aziz *et al.* 2019). ELISA kits have been developed to quantitatively assess CIRBP levels in peripheral blood, paving the way for clinical trials of CIRBP as a potential sepsis diagnostic marker (Zhou *et al.* 2015).

The significance of extracellular CIRBP as a critical inflammatory mediator has been studied in several inflammatory diseases and immune cells, including macrophages, lymphocytes, neutrophils, and dendritic cells (De Leeuw *et al.* 2007, Qiang *et al.* 2013). Additionally, the effects of extracellular CIRBP on endothelial and epithelial cells have also been investigated (Ran *et al.* 2016; Yang *et al.* 2016).

Once extracellular CIRBP is present, it binds to toll-like receptor 4 (TLR4) and the myeloid differentiation factor 2 (MD2), activating the NF-κB pathway (Aziz *et al.* 2019; Bolourani *et al.* 2021). Other membrane molecules may be involved in CIRBP-mediated signal transductions but require further investigation. After CIRBP binds to membrane molecules complexes, several signaling pathways are activated, including the NF-κB pathway (Aziz *et al.* 2019; Bolourani *et al.* 2021). The activation of such pathways leads to the induction and release of proinflammatory cytokines and chemokines (Qiang *et al.* 2013).

The role of CIRBP in reproductive physiology

In mammals, physiological hypothermia within the scrotum is required for proper spermatogenesis. CIRBP binds and stabilizes mRNAs related to male infertility (Xia *et al.* 2012). Additionally, CIRBP has a growth-stimulatory effect in spermatogenesis not limited to regulating mRNA stability (Masuda *et al.* 2012). CIRBP also prevents and reduces the damage of cryptorchidism (Zhou *et al.* 2009) and testicular torsion (Xia *et al.* 2013), and may exert a key function in heat stress-induced testicular spermatogenic cell injury (Cheng *et al.* 2019; Xu *et al.* 2020; Liu *et al.* 2021). In females, however, the studies about CIRBP functions are limited.

The first description of CIRBP in oocytes was in 2000 (Matsumoto *et al.* 2000). This report demonstrates the presence of a major RNA-binding protein in the cytoplasm of *Xenopus laevis* oocytes, called xCIRP2, a mammalian CIRBP homolog protein (Matsumoto *et al.* 2000). It was discovered that xCIRP2 is associated with ribosomes, suggesting that xCIRP2 participates in translational regulation of specific mRNAs in *Xenopus* oocytes (Matsumoto *et al.* 2000).

Therefore, being xCIRP2 homologous to the mammalian CIRBP (Matsumoto *et al.* 2000), CIRBP may regulate the mammalian oocyte translation modulating the ribosome function. Although a putative association of xCIRP2 and ribosomes has been demonstrated in *Xenopus* oocytes, the cellular roles of the cold-inducible proteins in oocytes are unknown. What is clear is that in response to mild hypothermia, mammalian CIRBP expression increases (Nishiyama *et al.* 1997); however, exposed to cold treatments, no expression changes of xCIRP2 were reported in *Xenopus* oocytes (Matsumoto *et al.* 2000).

Several reports had used the expression of CIRBP as a cryoinjury marker after cryoconservation of oocytes and embryos (Shin *et al.* 2011; Wen *et al.* 2014; Jo *et al.* 2015). However, CIRBP has been proposed as a potential candidate to increase the oocyte vitrification cryotolerance due to its role in cell survival and anti-apoptotic cascades (Zhu *et al.* 2016). In this regard, CIRBP has been linked with enhancing the developmental competence of mature yak vitrified/warmed oocytes (Pan *et al.* 2015). Because of the close evolutionary relationship between the yak (*Bos grunniens*) and the cow (*Bos taurus*), similar results may be expected with vitrified/warmed bovine oocytes using analogous approaches.

The female reproductive tract is in constant change, and it must adjust to the physiological demands of each reproductive stage (Forde *et al.* 2011). The estrous cycle is one of the most critical reproductive events in females throughout all their reproductive life (Estill 2015). The hormonal modulation during the estrous cycle causes several tissular alterations along the female reproductive tract to set up future reproductive events (Brenner and West 1975). The transcriptome regulation of the reproductive tissue has long been considered to be the inductor of such physiological adaptations (Brenner and West 1975). Several endometrial transcriptome alterations have been reported in cattle at different stages of the estrous cycle (Forde *et al.* 2011). The post-transcriptional control of RNAs is an essential regulatory step in gene expression (Änkö 2018). As an RNA-binding protein and regulator protein, CIRBP participates in various cellular functions, including cell survival and anti-apoptotic cascades, regulation of proliferation and differentiation, carcinogenesis, and inflammatory responses (Zhu *et al.* 2016; Liao *et al.* 2017; Zhong and Huang 2017). However, little is known about the CIRBP expression changes in response to the estrous cycle. In this regard, CIRBP has been involved in the cyclic changes of the endometrial cells in humans and seems to be negatively associated with the endometrial gland proliferative activity (Hamid *et al.* 2003).

Another important event in the female reproductive tract is the interaction between the male gametes and secretions and these tissues. This interaction induces protein and mRNA expression changes in endometrial and oviductal tissues of several species (Orihuela *et al.* 2001; Bauersachs *et al.* 2003; Fazeli *et al.* 2004; Georgiou *et al.* 2007; Almiñana *et al.* 2014; Álvarez-Rodríguez *et al.* 2020; Gardela *et al.* 2020a; Gardela *et al.* 2020b), modulating vital process in reproduction (i.e., angiogenesis, sperm storage and selection, oviduct contractility, oocyte transportation, and preimplantation embryo development). Additionally, after mating, sperm and seminal components induce an immune response to establish a suitable environment for the subsequent reproductive events (Hansen 2011; Katila 2012). Related to inflammation, CIRBP is linked to immune responses and modulates inflammatory processes (Zhu *et al.* 2016; Liao *et al.* 2017; Zhong and Huang 2017). Nevertheless, if CIRBP expression changes occur along the female reproductive tract in response to the male-female interaction is still unexplored.

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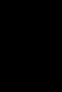
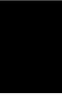
HYPOTHESIS



The optimal protocol for bovine oocyte vitrification remains to be developed. Among the several strategies that have been implemented to improve the outcomes of bovine oocyte vitrification, submitting the oocytes to sublethal stressors is a promising approach. Nevertheless, mild hypothermia sublethal stress has been neglected as a potential strategy. A more detailed understanding of the CIRBP regulation in oocytes and cumulus cells may help define new methods for improving bovine oocyte vitrification. In that sense, identifying methods to increase CIRBP expression in bovine oocytes and cumulus cells before vitrification could maximize the potential roles of CIRBP in cell survival cascades, thus improving the outcomes of bovine oocyte vitrification.

Additionally, its RNA chaperone and gene regulation activity make CIRBP an interesting candidate to study its modulation during the reproductive physiological processes. Increasing the knowledge about CIRBP modulation in the female reproductive tract during different reproductive events, including the estrous cycle and the male-female interaction, may be crucial to understanding the mechanisms behind the reproduction process.

OBJECTIVES



The general objective of this thesis is to study CIRBP as a potential molecule to improve bovine oocyte vitrification and determine its modulation during physiological changes in the female reproductive tract.

The specific objectives are as follows:

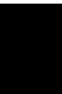
1. To evaluate whether the exposure of bovine cumulus-oocyte complexes to sublethal mild hypothermia during *in vitro* maturation may represent a potential strategy to increase CIRBP expression in oocytes and cumulus cells.

2. To assess the supplementation of exogenous CIRBP and the small molecule zr17-2 during *in vitro* maturation of bovine cumulus-oocyte complexes as an approach to increase *CIRBP* mRNA expression in oocytes and cumulus cells prior to vitrification.

3. To determine the endometrial and ampullary changes in CIRBP expression during the stages of the bovine estrous cycle.

4. To examine whether the male-female interaction modulates *CIRBP* mRNA expression in the reproductive tract of peri-ovulatory sows.

CHAPTER I



Induction of CIRBP expression by cold shock on bovine cumulus–oocyte complexes

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This chapter has been presented as an oral communication in the 15th International Congress of the Spanish Association for Animal Reproduction (AERA) in Toledo, Spain (9th November 2019). Additionally, this chapter has been published in *Reproduction in Domestic Animals.*

(Reprod. Domest. Anim. 2019;54(S4):82-85. doi: 10.1111/rda.13518)

Abstract

The aim of this study was to induce the cold-inducible RNA-binding protein (CIRBP) expression on cumulus–oocyte complexes (COCs) through exposure to a sub-lethal cold shock and determine the effects of hypothermic temperatures during the *in vitro* maturation of bovine oocytes. Nuclear maturation, cortical granule redistribution and identification of cold-inducible RNA-binding protein (CIRBP) were assessed after 24 h of *in vitro* maturation of control (38.5°C) and cold-stressed oocytes (33.5°C). The presence of CIRBP was assessed by Western blot in COCs or denuded oocytes and their respective cumulus cells. Based on the odds ratio, cold-stressed oocytes presented higher abnormal cytoplasmic distribution of cortical granules and nuclear maturation than the control group. Although CIRBP was detected in both control and cold-stressed groups, cold-stressed COCs had 2.17 times more expression of CIRBP than control COCs. However, when denuded oocytes and cumulus cells were assessed separately, CIRBP only was detected in cumulus cells in both groups. In conclusion, cold shock induced CIRBP expression, but it negatively affected nuclear maturation and cortical granule distribution of bovine oocytes. Moreover, the expression of CIRBP was only identified in cumulus cells but not in oocytes.

Keywords: CIRBP protein; cold-shock response; domestic cow; *in vitro* oocyte maturation

Introduction

Cryopreservation of germplasm has become an essential part of the assisted reproductive techniques. These technologies allow conservation of animal genetic resources and preservation of the fertility in women. However, there are still some difficulties regarding the application of the cryopreservation methods on oocytes due to the large size and marked sensibility to cooling injuries of these cells (Sprícigo *et al.* 2012).

Different strategies have been used to improve cryotolerance in mammalian oocytes through a temporary increase of general adaptation induced by sub-lethal stressors (Pribenszky *et al.* 2010) such as high hydrostatic pressure (Gu *et al.* 2017) and heat stress (Vendrell-Flotats *et al.* 2017). In the same way, we hypothesized that exposure to low temperatures prior vitrification may induce cryotolerance in mammalian gametes and embryos.

The exposure to mild hypothermic temperatures induces the expression of cold-shock proteins (Liao *et al.* 2017). CIRBP, also called CIRP and A18 hnRNP, is a constitutively expressed cold-shock protein highly conserved among different species whose expression is present in a large variety of tissues and cells, including the ovaries among others (Zhong and Huang 2017). CIRBP is involved in several cellular processes such as cellular proliferation and cell survival and it is involved in anti-apoptotic and anti-senescence pathways (Liao *et al.* 2017; Zhong and Huang 2017). These findings suggest that the induction of CIRBP during *in vitro* maturation (IVM) of oocytes could improve cryotolerance to vitrification procedures. For that reason, the aim of this study was to determine the responsiveness of bovine oocytes (*Bos taurus*) to differentially express CIRBP through hypothermic temperatures as a preliminary study before testing predicted CIRBP protective effects against oocyte vitrification.

Materials and methods

All experiments were performed according to the principles and guidelines of the Ethics Committee on Animal and Human Experimentation from the *Universitat Autònoma de Barcelona*.

Experimental design

Cumulus-oocyte complexes (COCs) were randomly distributed in two groups: control (C) and cold-stressed groups (CS). After 24 h of IVM, oocytes were fixed in paraformaldehyde (PFA) to

evaluate nuclear maturation and cytoplasmic distribution of cortical granules (CGs). Additionally, COCs or denuded oocytes and their respective cumulus cells from both experimental groups were frozen at -20°C for Western blot analysis. Three independent biological replicates were performed in total.

In vitro maturation

COCs were collected by aspirating follicles from heifer ovaries after collecting them at a local slaughterhouse. After 3 washes in phosphate buffer saline supplemented with 0.5 mg/mL bovine serum albumin, 1 mg/mL glucose, 36 µg/mL pyruvate and 0.05 mg/mL gentamycin, groups of 50 oocytes were randomly placed in 500 µL maturation medium in four-well dishes and cultured for 24 h at 38.5°C (C) or 33.5°C (CS) in independent incubators under an atmosphere of 5% CO_2 in humidified air. The maturation medium was composed by TCM-199 supplemented with 10% foetal calf serum and 10 ng/ml epidermal growth factor.

Assessment of nuclear maturation and cortical granule distribution

COCs were denuded of cumulus cells by gentle pipetting. Nuclear maturation was assessed as the percentage of oocytes that have reached the metaphase II stage by checking the extrusion of the first polar body. The zona pellucida was dissolved using a solution containing 0.4% pronase for 8 min. Oocytes were then fixed in 4% PFA (45 min, room temperature), permeated (0.3% Triton-X100, 30 min, room temperature) and stained (100 µg/mL fluorescein isothiocyanate-labeled *Lens culinaris* agglutinin) as previously described by Andreu-Vázquez *et al.* (2010). Oocytes were transferred to mounting medium containing DAPI (Vector labs, Burlingame, CA, USA) and coverslipped. CGs distribution was classified into four patterns according to the classification of Hosoe & Shioya (1997) modified by Andreu-Vázquez *et al.* (2010) (pattern I: distribution in clusters - immature CGs distribution; pattern II: individually dispersed and partially clustered - incomplete CGs distribution; pattern III: distributed beneath the plasma membrane - optimal CGs distribution; pattern IV: no CGs - over matured).

Western blotting for CIRBP

Western blotting (WB) was performed following the described protocol by Alvarez-Rodriguez *et al.* (2019). Briefly, COCs or denuded oocytes and their respective cumulus cells were homogenized by sonication in commercial lysis buffer (RIPA) at 4°C. Protein concentration was determined by the DC™ Protein Assay kit (Bio-Rad), with bovine serum albumin as standard. Then, 25 µg of each sample were mixed with 4× sample buffer and heated for 10 min at 70°C. Extractions were loaded into 4%-20% SDS-PAGE gels and transferred to polyvinylidene

difluoride membranes. For protein identification, membranes were blocked at room temperature for 60 min and incubated overnight at 4°C with rabbit monoclonal anti-CIRBP antibody [EPR18783] (ab191885, Abcam) at dilution 1/500. To standardize the results, a polyclonal IgG anti-α-Tubulin antibody (Sigma) was used at a dilution 1/1,000 in the same membranes. To visualize immunoreactivity, membranes were incubated 60 min at room temperature with secondary antibody anti-rabbit horseradish peroxidase conjugated (31460, Pierce Biotechnology) at dilution 1/10,000. After scanning by FluorChem® HD2 (Alpha Innotech), optical density was quantified by ImageJ Software.

Statistical analysis

The R Software (version 3.4.4) was used for data analysis. Replicate (1-3), group (C and CS), extrusion of first polar body (matured and non-matured) and CGs distribution (pattern I and III) were recorded for each oocyte. Three logistic regression analyses were performed in total using the nuclear maturation state or the CGs distribution data as dependent variables (0 and 1) in each individual analysis. Replicate and group were used as independent factors in each analysis. Intensity of CIRBP bands in WB were analyzed by t-test comparing C with CS groups.

Results

Based on the odds ratio, the likelihood for an oocyte of showing CGs distribution pattern I (immature CGs distribution) was 9.75 times higher for CS than for C ($p < 0.05$). For CGs distribution pattern III (optimal CGs distribution), the likelihood to show non-optimal distribution pattern was 5.6 times greater for CS than for C ($p < 0.05$). The risk to undergo anomalous nuclear maturation was 2.72 times higher in CS oocytes than C ones ($p < 0.05$) (Figure **1**).

CIRBP expression was detected in both C and CS groups. Significantly higher ($p < 0.05$) levels of intensity were observed in CIRBP bands of CS compared with C in COCs analysis (Figure **2** and Figure **3**). When oocytes were denuded, no expression of CIRBP was detected in oocytes while their respective cumulus cells showed CIRBP expression in both C and CS groups (Figure **2**).

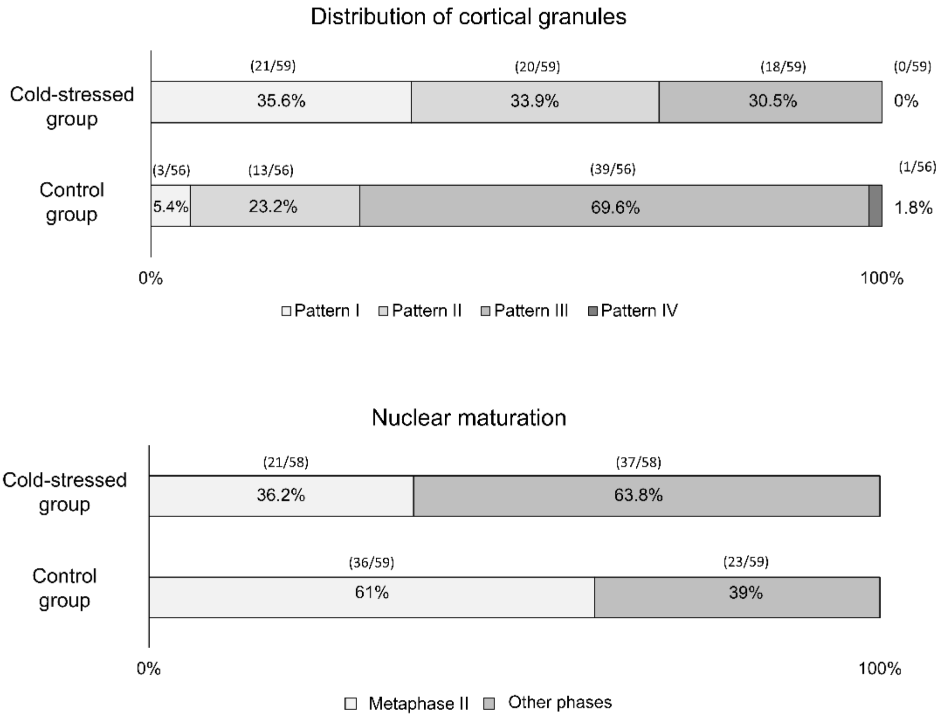

Figure 1. Distribution of cortical granules (CGs) and nuclear maturation of cold-stressed oocytes (n = 59 and n = 58, respectively) and control oocytes (n = 56 and n = 59, respectively) during 24 h of *in vitro* maturation. CG distribution was distributed into four patterns according to the classification of Hosoe and Shioya (1997) modified by Andreu-Vázquez *et al.* (2010) (pattern I: distribution in clusters - immature CG distribution; pattern II: individually dispersed and partially clustered - incomplete CG distribution; pattern III: distributed beneath the plasma membrane - optimal CG distribution; pattern IV: no CGs - over matured). Nuclear maturation was classified as the extrusion of the first polar body.

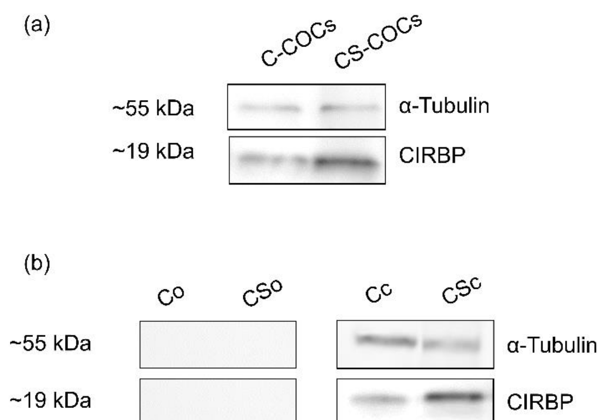

Figure 2. Analysis of the presence of CIRBP (19 kDa) by Western blotting (WB) in cumulus-oocyte complexes (COCs) and denuded oocytes and their respective cumulus. Oocytes were *in vitro* matured at 38.5°C (control group) or at 33.5°C (cold- stressed group). Membrane (**a**): WB of COCs; membrane (**b**): WB of denuded oocytes and their respective cumulus cells. C-COCs: control COCs, CS-COCs: cold- stressed COCs, Co: control oocytes, Cc: control cumulus cells, CSo: cold- stressed oocytes, CSc: cold-stressed cumulus cells.

Discussion and conclusions

To our knowledge, this is the first study to describe the differential expression of CIRBP on bovine COCs after IVM in sub-lethal cold-shock-induced conditions as well as its effect on oocyte nuclear maturation and cytoplasmic distribution of CGs. According to our results, cold shock appears to negatively affect the optimal competence of oocytes regarding nuclear maturation and cytoplasmic CGs distribution. In addition, cold shock induced an increase of CIRBP expression on COCs. The increase of CIRBP in cumulus cells could play important roles in cryoprotective protection of oocytes through the interaction between cumulus cells and oocytes (Komatsu and Masubuchi 2018). However, little is known about the relationship between CIRBP expression and the developmental competence of bovine vitrified-warmed oocytes. In this way, the developmental competence of vitrified-warmed yak oocytes (*Bos grunniens*) was improved by an increase of CIRBP (Pan *et al.* 2015). Taking together, new approaches should be performed to clarify the role of CIRBP on bovine COCs. Moreover, further studies are needed to apply the differential expression of CIRBP in cumulus cells into an effective tool for improving vitrification cryotolerance minimizing the intrinsic negative effects of cold shock during IVM of bovine oocytes.

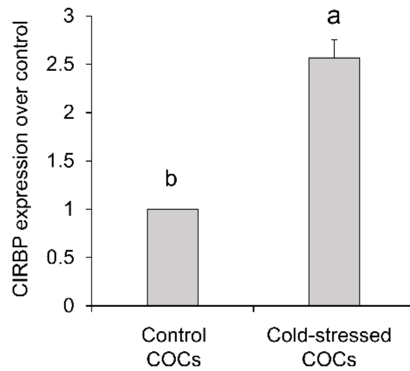

Figure 3. Relative expression (mean ± SD) of CIRBP protein in cumulus of bovine cumulus–oocyte complexes (COCs) in control and cold-stressed groups. Three independent blots were used for relative quantification. The control group matured at 38.5°C was used as calibrator. Different letters on the bars indicate values that differed significantly ($p < 0.05$).

Acknowledgements: Project AGL2016-79802-P and AGL2016-81890-REDT supported this study. JG is recipient of a FI grant (2018 FI_B 00236). MRC is funded by FPU2015/06029. MAR is supported by IJCI-2015-24380. We thank the staff from Mercabarna slaughterhouse for the samples provided and Sonia Pina-Pedrero for her technical assistance during WB analysis.

Conflict of Interest Statement: None of the authors have any conflict of interest to declare.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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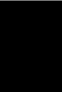
CHAPTER II



Mild hypothermia and vitrification increase the mRNA expression of cold-inducible proteins in bovine oocytes and cumulus cells

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This chapter is under review in *Theriogenology*.

Abstract

The cold-inducible RNA-binding protein (CIRBP) assists cells in adapting to new environmental conditions stabilizing specific mRNAs and promoting their translation. CIRBP participates in anti-apoptotic and anti-senescence processes, and its expression is induced by mild hypothermia, which may be advantageous to oocytes during vitrification. Several newly discovered small molecules, like zr17-2, mimic the effects of cold temperatures by increasing the expression of CIRBP at normothermia. This study aimed to evaluate the mRNA changes of a group of cold-inducible protein-encoding and apoptotic genes in response to exogenous supplementation of zr17-2 (experiment 1) or CIRBP (experiment 2) *in vitro* matured bovine oocytes and their cumulus cells. In experiment 1, cumulus-oocyte complexes (COCs) were randomly exposed to three concentrations of zr17-2 (1, 10, 100 μM) during 24 h of *in vitro* maturation (IVM) under normothermia (38.5°C) or mild hypothermia (34°C) culture conditions. In experiment 2, COCs were randomly exposed to three concentrations of CIRBP (2, 4, and 6 μg/mL) or subjected to mild hypothermia (34°C), followed by oocyte vitrification/warming after 20 h of IVM. The quantification of the selected gene transcript expression was performed separately in oocytes and cumulus cells by quantitative real-time PCR. We show that oocytes and cumulus cells exhibited similar mRNA expression responses to mild hypothermia and vitrification. However, minor differences were observed when COCs were exposed to exogenous supplementation with zr17-2 and CIRBP. In conclusion, the alterations observed in the mRNA expression in these experimental conditions may impact the quality of the cumulus-oocyte complexes in terms of vitrification and sublethal hypothermia challenges. In this sense, our results should complement other oocyte quality assessments for its application in future assisted reproductive techniques in the bovine species, including improving oocyte cryotolerance to vitrification.

Keywords: *in vitro* maturation; hypothermia mimetic; cold-inducible RNA-binding protein; cow

Introduction

Oocyte cryopreservation is a valuable methodology for preserving genetic resources and contributes to developing assisted reproductive techniques (Zhou *et al.* 2010). On the other hand, cryopreservation may damage the structure of the oocyte and chromosomes, as well as the mitotic spindle (Sprícigo *et al.* 2012; Chaves *et al.* 2017; Pitchayapipatkul *et al.* 2017). During this process, the ultra-low temperatures affect mitochondrial activity, affecting apoptotic pathways (Zhang *et al.* 2020), inducing premature cortical granule extrusion, and reducing successful fertilization chances (Ghetler *et al.* 2006).

In mammalian cells, exposure to mild hypothermia (34°C) reduces the metabolism and the overall protein abundance, while the expression of a small group of homologous glycine-rich mRNA-binding proteins, known as cold-inducible proteins (CIPs), increases under this condition (Derry *et al.* 1995; Danno *et al.* 1997; Nishiyama *et al.* 1997; Tong *et al.* 2013; Rzechorzek *et al.* 2015; Fujita *et al.* 2017). The CIPs bind to mRNA, acting as regulatory molecules that play an essential role in RNA post-transcriptional control (Lunde *et al.* 2007). They are upregulated in response to mild hypothermia and a variety of other cellular stressors, including osmotic stress, ultraviolet radiation, or hypoxia (Zhu *et al.* 2016).

The cold-inducible RNA-binding protein (CIRBP) is a constitutively and ubiquitously expressed CIP considered a key component of the cellular response to moderate cold stress (Liao *et al.* 2017). CIRBP has been identified in multiple tissues and species (Su *et al.* 2004), including the bovine ovary and testicle (Gardela, García-Sanmartín, *et al.* 2019). Although only a few body tissues are exposed to hypothermia in physiological conditions, the widespread expression of CIRBP (Su *et al.* 2004) may suggest that it is likely involved in other relevant cellular processes at normothermia. Primarily located in the nucleus, CIRBP controls RNA transcription and processing, while in the cytoplasm, CIRBP regulates mRNA translation and turnover (Nishiyama *et al.* 1997; Zhong and Huang 2017). Eventually, CIRBP can be released into the extracellular space (Qiang *et al.* 2013). Once extracellular CIRBP is present, it binds to toll-like receptor 4 (TLR4) and the myeloid differentiation factor 2 (MD2), activating several signaling pathways including the NF-κB pathway (Aziz *et al.* 2019; Bolourani *et al.* 2021). The activation of such pathways via CIRBP leads to an induction and release of proinflammatory cytokines and chemokines, including the IL-1β (Brochu *et al.* 2013; Qiang *et al.* 2013). However, if IL-1β is modulated during *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes (COCs) in the presence of CIRBP is still unknown.

Other CIPs, as the RNA-binding motif protein 3 (RBM3) and the serine and arginine-rich splicing factor 5 (SRSF5) (Derry *et al.* 1995; Fujita *et al.* 2017), also bind to particular mRNAs, stimulating their translation, regulating their half-life, controlling their expression potential, and determining their final functions (Wellmann *et al.* 2004; Liu *et al.* 2013). Moreover, CIRBP and RBM3 bind to specific mRNAs involved in cell survival and anti-apoptotic cascades (Zhu *et al.* 2016), being potential candidates to improve oocyte cryotolerance to vitrification. Because oocyte vitrification can induce apoptosis (García-Martínez *et al.* 2020; Shirzeyli *et al.* 2021), the study of BCL2 (anti-apoptotic gene) (Yang *et al.* 1997), BAX (pro-apoptotic gene) (Yang and Rajamahendran 2002), and its ratio is helpful to investigate the effects of vitrification on oocytes and cumulus cells.

A temporary increase in general adaptability generated by controlled exposure to sub-lethal stressors has been used to improve cryotolerance in mammalian oocytes and embryos using various strategies (Pribenszky *et al.* 2010), such as heat stress (Vendrell-Flotats *et al.* 2017) or high hydrostatic pressures (Gu *et al.* 2017). However, little attention has been given to mild hypothermia as a sub-lethal stressor to enhance stress tolerance. In this sense, we have previously reported an increase in CIRBP expression on bovine COCs exposed to mild hypothermia (33.5°C) (Gardela, Ruiz-Conca, *et al.* 2019). However, detrimental effects on oocyte nuclear and cytoplasmic maturation rates were reported due to the non-optimal temperature conditions during the IVM (Gardela, Ruiz-Conca, *et al.* 2019). Furthermore, some small molecules (including zr17-2) have been detected, capable of mimicking the effect of hypothermia in the absence of cold stimulus (Coderch *et al.* 2017), being good candidates for their application to improve cell cryotolerance.

In the present study, we hypothesized that oocytes and cumulus cells might modify the expression of selected genes during the IVM in response to the exogenous supplementation with zr17-2 and CIRBP directly added to the culture medium. For this purpose, we aim to test the effect of exogenous supplementation with zr17-2 and CIRBP during the IVM of bovine COCs and whether this procedure implies upregulation of mRNA expression of the three CIPs included (*CIRBP*, *RBM3*, and *SRSF5*), *BCL2*, and *IL-1β*, and downregulation of *BAX* mRNA expression. Here we will determine the mRNA changes on three CIPs, apoptotic-related transcripts (*BAX* and *BCL2*, and their ratio), and the pro-inflammatory interleukin 1β (*IL-1β*) induced by mild hypothermia and vitrification in both oocytes and cumulus cells.

Materials and methods

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Bovine COCs recovery and IVM

Bovine ovaries were obtained from a local slaughterhouse (*Escorxador de Sabadell*, Barcelona, Spain) and transported to the laboratory at 35-37°C in 0.9% saline solution. The ovaries were collected from cycling non-gravid heifers in follicular phase. Immature COCs were aspirated from ovarian follicles between 6-8 mm in diameter using an 18-gauge needle attached to a low-pressure vacuum pump. Only COCs presenting more than three compact layers of cumulus cells and a homogeneous cytoplasm were selected for the study. The selected COCs were randomly allocated in groups of 50 oocytes in 500 µL of maturation medium in 4-well dishes (Nalge Nunc International, Rochester, NY, USA) and cultured following the experimental design. The maturation medium was composed of TCM199 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.

Experiment 1: hypothermia mimetic zr17-2 supplementation

In experiment 1, COCs were randomly distributed in groups of 50 oocytes in 4 experimental groups: exposed to control conditions or exposed to three different concentrations of zr17-2 (1, 10, 100 µM). These 4 experimental groups were incubated for 24 h in a humidified atmosphere containing 5% CO_2 at two different temperature conditions, 38.5°C or 34°C. After 24 h of IVM, COCs were washed and denuded by gently pipetting (PIPETMAN ® P100L, Gilson, Spain) in phosphate buffer saline medium. Oocytes were harvested and the remaining media after denudation was centrifuged (500× *g*, 3 min, room temperature) to obtain the cumulus cells of the oocytes on each experimental group. The supernatant was discarded and the cryotube containing the cell pellet of each group was immediately plunged in liquid nitrogen. Oocytes of each group (n = 25 oocytes/experimental group and replicate) were transferred to cryotubes using the minimum possible volume of media and directly immersed in liquid nitrogen. All samples were kept at -80°C until further analysis. The experiment was performed three times.

Experiment 2: exogenous CIRBP supplementation

In experiment 2, COCs were randomly distributed in groups of 50 oocytes in 5 experimental groups: exposed to standard bovine IVM conditions (38.5°C, 5% CO_2, in humidified

atmosphere), exposed to three different concentrations of exogenous CIRBP (2, 4, and 6 μ/mL), or exposed to mild hypothermia (34°C, 5% CO_2, in humidified atmosphere). After 20 h of IVM, half of the COCs on each experimental group were vitrified and ulteriorly warmed (n = 25 oocytes/experimental condition and replicate). Vitrified/warmed oocytes were returned for 4 h to the maturation dishes to complete 24 h of IVM. After finishing the 24 h of IVM, COCs (n = 25/experimental group and replicate) were denuded by gently pipetting to separate oocytes from cumulus cells, as before described. The experiment was performed three times.

Oocyte vitrification and warming

After 20 h of IVM, COCs were partially denuded by gentle pipetting until a few layers of cumulus cells remained. Following a modified vitrification protocol (Kuwayama *et al.* 2005), oocytes were transferred into an equilibrium solution (TCM199 with HEPES supplemented with 20% (v/v) FBS, 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (Me_2SO)) for 9 min at room temperature (20-22°C). After being equilibrated, oocytes were transferred into a vitrification solution (TCM199 with HEPES supplemented with 20% (v/v) FBS, 15% (v/v) EG, 15% (v/v) Me_2SO, and 0.5 M sucrose) for 30 s. Groups of 5-6 oocytes were then picked up in a small volume of vitrification solution, placed on top of the polypropylene strip of a Cryotop device (Kitazato Supply Co, Fujinomiya, Japan), and immediately plunged in liquid nitrogen.

For warming, the polypropylene strip of the Cryotop device was immersed directly into TCM199 with HEPES supplemented with 20% (v/v) FBS containing 1 M sucrose at 37°C for 5 min. Recovered oocytes were washed for 5 min in decreasing solutions of sucrose (0.5 M, 0.25 M, and 0 M sucrose in TCM199 with HEPES). Once warming was finished, oocytes were returned to the maturation dishes allowing them to mature for two additional hours.

RNA extraction and cDNA synthesis

Total RNA from oocytes and cumulus cells was extracted using the commercial miRNeasy Mini Kit (Qiagen, Barcelona, Spain) following the manufacturer's instructions. The RNA concentration was determined using the Thermo Scientific NanoDropTM 2000 (Fisher Scientific, Madrid, Spain). Synthesis of cDNA was performed using the High-Capacity RNA-to-cDNA™ Kit (Fisher Scientific, Madrid, Spain) according to the manufacturer's instructions. The cDNA obtained was stored at -20°C for subsequent analysis.

Quantitative real-time PCR (qPCR)

Relative quantification of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* transcripts was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with *G3PDH* as a housekeeping gene for normalization. Data analysis was performed using the CFX Maestro software version 3.3.3 (Bio-Rad Laboratories, Kabelsketal, Germany). Commercial gene-specific qPCR primers for bovine species were used (PrimePCR™SYBR® Green Assay, Bio-Rad Laboratories, Kabelsketal, Germany). The product sizes for each primer pair were confirmed by loading the amplicons in an agarose gel using a gel imaging system (ChemiDoc XRS+ System, BioRad Laboratories, Inc., Kabelsketal, Germany).

Data analyses

Each experiment was repeated three times. The relative mRNA expression data of the genes included in the study were analyzed by R software version 3.6.1 (R Core Team 2019) with *nlme* (Pinheiro *et al.* 2020) 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 1, the experimental group and the temperature during IVM (34°C or 38.5°C) were included as fixed effects and the replicates as the random part of the LME. Pairwise comparisons were adjusted by the Sidak test.

For experiment 2, the experimental group and 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: hypothermia mimetic zr17-2 supplementation

The relative mRNA expression of selcted transcripts showed that mild hypothermia induced most of the changes observed in *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, and *BAX* mRNA expression, both in oocytes (Figure **1a**) and cumulus cells (Figure **1b**).

Mild hypothermia upregulated *CIRBP* and *RBM3* mRNA expression in oocytes and cumulus cells ($p < 0.05$). *SRSF5* mRNA expression was upregulated in cumulus cells exposed to mild

hypothermia culture conditions ($p < 0.05$), but no differences were observed in oocytes. *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at different concentrations of zr17-2 compared to the control group ($p < 0.05$). Besides, *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at 100 μM zr17-2 compared to all the experimental groups at normothermia ($p < 0.05$). Additionally, *RBM3* mRNA expression was upregulated in oocytes exposed to mild hypothermia at 10 μM zr17-2 compared to oocytes exposed to normothermia at 10 μM zr17-2 and IVM control conditions ($p < 0.05$).

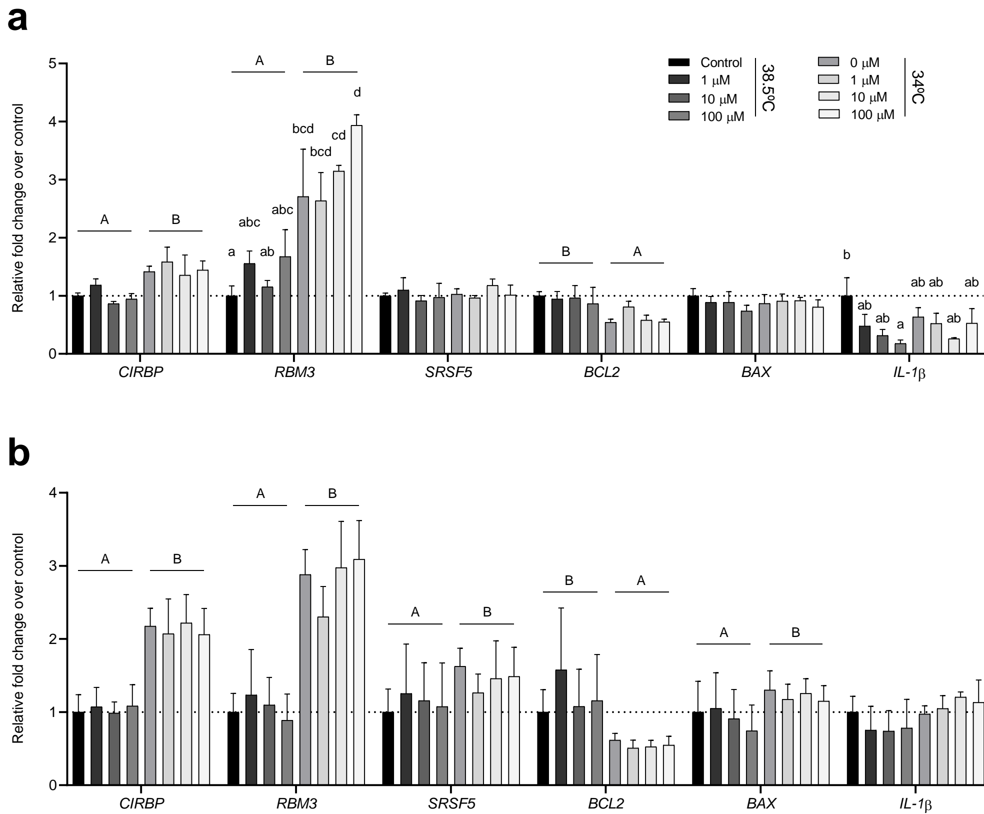

Figure 1. Relative mRNA expression of selected genes in oocytes and cumulus cells of experiment 1. Relative mRNA expression ± SEM of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* in oocytes (**a**) and cumulus cells (**b**) of experimental groups in experiment 1. Supplementation with four concentrations of zr17-2 (0, 1, 10, 100 μM) incubated at 38.5°C or 34°C, 5% CO_2 in humidified atmosphere. Different lowercase-letters represent statistical differences between experimental groups. Different uppercase-letters represent statistical differences between culture temperature conditions. The control group (0 μM zr17-2 incubated at 38.5°C, 5% CO_2 in humidified atmosphere) was established as the reference group.

The mRNA expression of anti-apoptotic *BCL2* was downregulated in oocytes and cumulus cells cultured under mild hypothermia conditions ($p < 0.05$), whereas mRNA expression of the pro-apoptotic gene *BAX* was upregulated in cumulus cells cultured under mild hypothermia conditions ($p < 0.05$).

When cultured at normothermia, the relative mRNA expression of *IL-1β* was downregulated in oocytes by the 100 μM zr17-2 supplementation compared to the control group ($p < 0.05$).

Experiment 2: exogenous CIRBP supplementation

The relative mRNA expression of selected transcripts in Figure **2** shows that mild hypothermia induced most of the changes observed in the *CIRBP* and *RBM3* mRNA expressions, whereas the vitrification procedure induced changes in *SRSF5* mRNA expression.

CIRBP mRNA expression was upregulated in oocytes exposed to mild hypothermia (vitrified and non-vitrified) compared to 6 μg/mL CIRBP (non-vitrified), 2 μg/mL CIRBP (vitrified and non-vitrified), and vitrified control oocytes ($p < 0.05$). For cumulus cells, the *CIRBP* mRNA expression was upregulated in non-vitrified cells exposed to mild hypothermia compared to vitrified cumulus cells exposed to 2 μg/mL CIRBP ($p < 0.05$).

RBM3 mRNA expression was upregulated in oocytes exposed to mild hypothermia (vitrified and non-vitrified) compared to the rest of the experimental groups ($p < 0.05$). In non-vitrified cumulus cells exposed to mild hypothermia, *RBM3* mRNA expression was upregulated compared to the rest of the non-vitrified experimental groups and the vitrified cumulus cells exposed to 2 and 6 μg/mL of exogenous CIRBP ($p < 0.05$). Additionally, *RMB3* mRNA expression was upregulated in vitrified cumulus cells exposed to mild hypothermia compared to the rest of vitrified and non-vitrified treatments, excluding the non-vitrified cumulus cells exposed to mild hypothermia ($p < 0.05$).

SRSF5 mRNA expression in oocytes and cumulus cells was increased by the vitrification procedure ($p < 0.05$). Besides, *SRSF5* mRNA expression was upregulated in vitrified oocytes exposed to mild hypothermia compared to non-vitrified oocytes exposed to 4, 2 μg/mL CIRBP, and control conditions ($p < 0.05$). Additionally, SRSF5 expression was upregulated in vitrified oocytes exposed to 4 μg/mL CIRBP compared to non-vitrified oocytes exposed to 2 μg/mL CIRBP and control conditions ($p < 0.05$).

Vitrification and mild hypothermia induced most of the changes observed in the *BCL2* mRNA of oocytes Figure **2a** and cumulus cells Figure **2b**, respectively. In vitrified and non-vitrified cumulus cells exposed to mild hypothermia, the *BCL2* mRNA expression was downregulated compared to non-vitrified control cumulus cells and the vitrified and non-vitrified cumulus cells

exposed to 4 μg/mL of exogenous CIRBP ($p < 0.05$). On the other hand, the vitrification procedure induced *BAX* and *IL-1β* mRNA expression changes in the cumulus cells. Additionally, *BAX* mRNA expression was upregulated in vitrified oocytes exposed to 6 μg/mL CIRBP compared to vitrified oocytes exposed to mild hypothermia and non-vitrified oocytes exposed to 4 μg/mL CIRBP ($p < 0.05$).

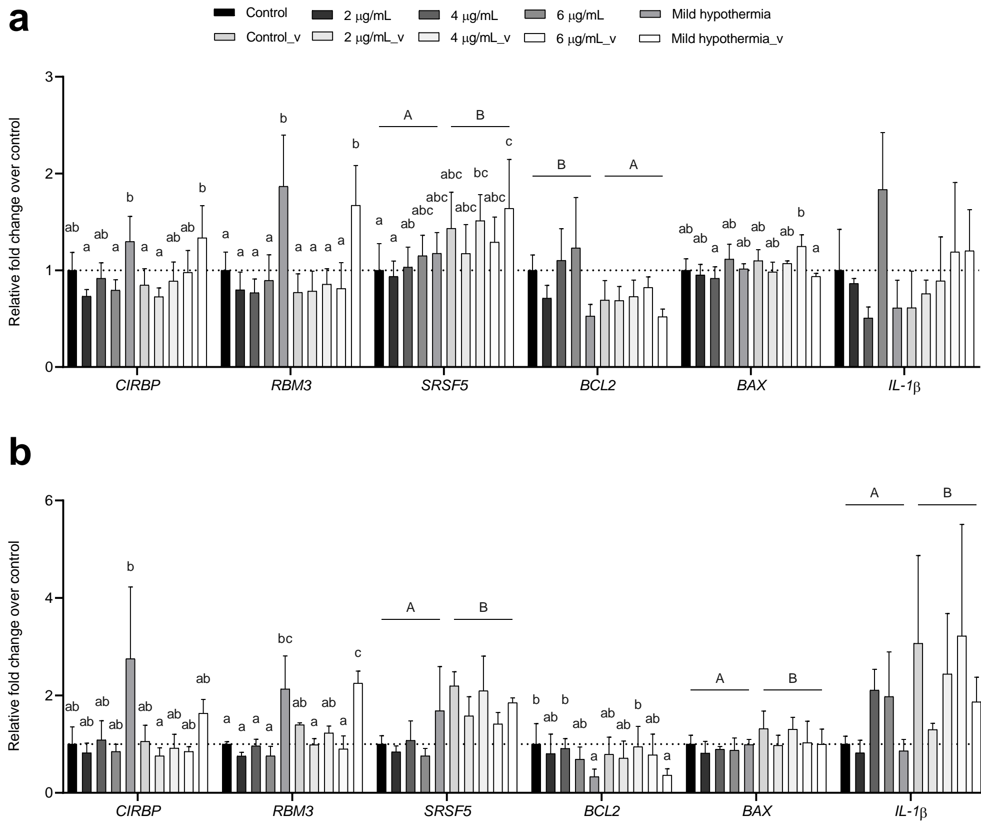

Figure 2. Relative mRNA expression of selected genes related in oocytes and cumulus cells of experiment 2. Relative mRNA expression ± SEM of *CIRBP*, *RBM3*, *SRSF5*, *BCL2*, *BAX*, and *IL-1β* in oocytes (**a**) and cumulus cells (**b**) of treatments in experiment 2. Control (38.5°C, 5% CO_2 in humidified atmosphere); three concentrations of exogenous CIRBP protein (2, 4, 6 μg/mL; 38.5°C, 5% CO_2 in humidified atmosphere); and mild hypothermia (34°C, 5% CO_2 in humidified atmosphere). Vitrified groups were represented with letter v. Different lowercase-letters represent statistical differences between treatments. Different uppercase-letters represent statistical differences between vitrified and non-vitrified groups. The control group was established as the reference group.

Apoptosis gene expression: BAX:BCL2 ratio

The relative BAX:BCL2 ratios were increased by mild hypothermia in experiment 1 (Figure **3a** and Figure **3b**) and vitrification in experiment 2 (Figure **3c** and Figure **3d**) ($p < 0.05$).

Additionally, in experiment 2, the ratio was increased in non-vitrified oocytes exposed to mild hypothermia compared to non-vitrified oocytes exposed to 4 μg/mL CIRBP and to control conditions ($p < 0.05$) (Figure **3c**). On the other hand, the ratio was increased in vitrified and non-vitrified cumulus cells exposed to mild hypothermia compared to the non-vitrified treatments ($p < 0.05$) (Figure **3d**). Besides, the ratio was increased in non-vitrified cumulus cells exposed to mild hypothermia compared to the rest of vitrified cumulus cells ($p < 0.05$), except for the vitrified cumulus cells exposed to mild hypothermia (Figure **3d**).

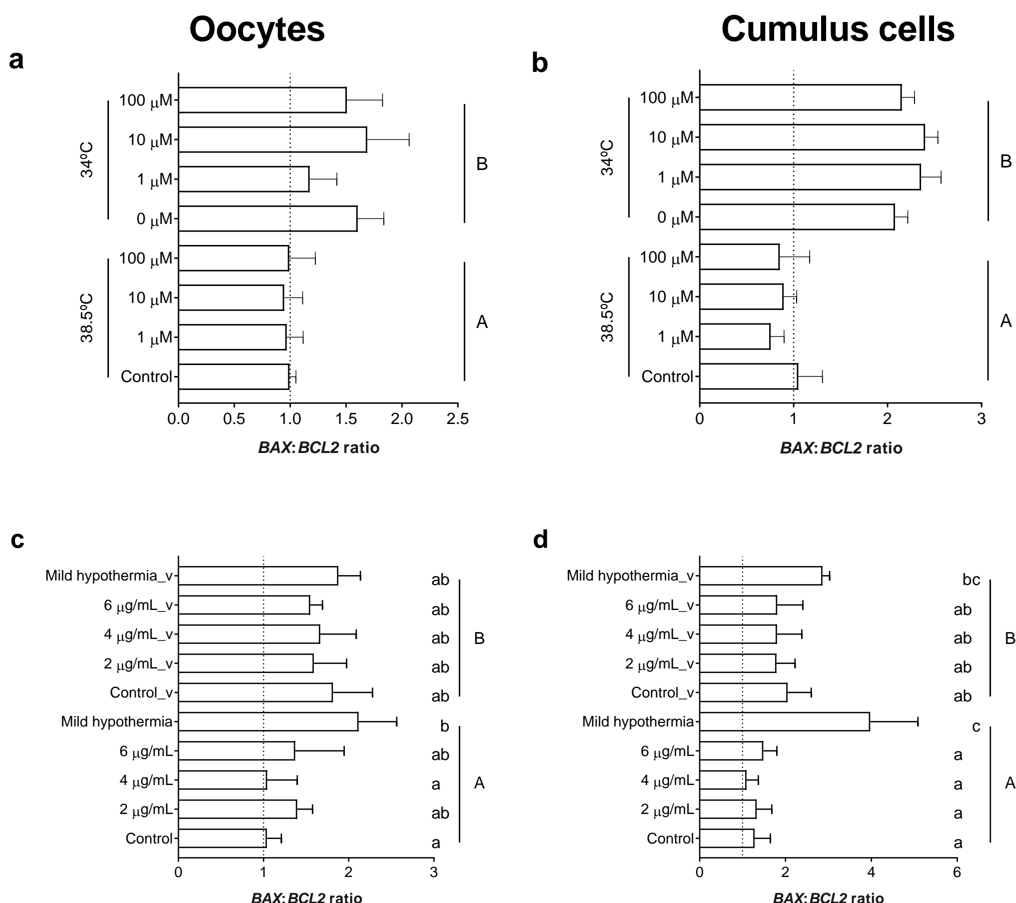

Figure 3. Relative BAX:BCL2 ratios in oocytes and cumulus cells of experiment 1 and 2. Relative BAX:BCL2 ratio ± SEM in oocytes (**a, c**) and cumulus cells (**b, d**) of experiment 1 (**a, b**) and experiment 2 (**c, d**). Experiment 1: supplementation with four concentrations of zr17-2 (0, 1, 10, 100 μM) incubated at 38.5°C or 34°C, 5% CO_2 in humidified atmosphere.

Experiment 2: control (38.5°C, 5% CO2 in humidified atmosphere); supplementation with three concentrations of exogenous CIRBP protein (2, 4, 6 µg/mL; 38.5°C, 5% CO_2 in humidified atmosphere); and mild hypothermia (34°C, 5% CO_2 in humidified atmosphere). Vitrified groups were represented with letter v. Different lowercase-letters represent statistical differences between experimental groups. Different uppercase-letters represent statistical differences between culture temperature conditions (experiment 1) or vitrified and non-vitrified groups (experiment 2). The control group was established as the reference group for each experiment.

Discussion

Different strategies have been used to improve cryotolerance in mammalian oocytes by increasing the general adaptability induced by sublethal stressors (Pribenszky *et al.* 2010). Mild hypothermia, on the other hand, has received little attention as an inductor of tolerance. Furthermore, the CIPs are potential candidates to mitigate the damage produced in oocytes during vitrification, mainly due to their involvement in cell survival and anti-apoptotic pathways (Zhu *et al.* 2016). This study has determined mRNA expression changes of CIPs and anti- and pro-apoptotic transcripts in oocytes and cumulus cells induced by mild hypothermia, vitrification, and exogenous zr17-2 and CIRBP supplementation.

Oocytes and cumulus cells exhibited similar mRNA expression responses to mild hypothermia and vitrification, but minor differences were observed when COCs were exposed to exogenous supplementation with zr17-2 and CIRBP. Our findings may suggest a common underlying mechanism of such observed response to mild hypothermia and vitrification in both types of cells. The bidirectional communication between oocytes and cumulus cells is essential to establish the optimal intrafollicular microenvironment, which controls the growth and maturation of the follicles to produce viable and competent oocytes (Dumesic *et al.* 2015; Russell *et al.* 2016). In addition, this complex communication may be involved in shared responses to ensure that both cells quickly adapt to the new environmental conditions. This fact suggests that the mRNA expression changes that occurred during the maturation of the oocyte may have potential effects during early embryo development (Russell *et al.* 2016). In this sense, the regulation of CIRBP expression in mature yak oocytes before vitrification may explain the improvement in its *in vitro* developmental competence after vitrification/warming (Pan *et al.* 2015).

Several CIPs have been previously identified; yet, two of them (CIRBP and RBM3) have been well-characterized since their discovery (Derry *et al.* 1995; Nishiyama *et al.* 1997). Both proteins regulate gene expression at the transcription level by binding to different transcripts, allowing the cell to respond quickly to environmental changes (Lleonart 2010). Here, we confirmed that the

mRNA expressions of *CIRBP* and *RBM3* were significantly induced by mild hypothermia in oocytes and cumulus cells, with or without an additional vitrification procedure. Even though oocytes were exposed to ultralow temperatures during vitrification, it seems that the brief exposure time to sub-physiological temperatures was not enough to induce the transcription of neither both CIPs when cells were previously long exposed to exogenous CIRBP and mild hypothermia. In contrast, previous studies have reported an increase of *CIRBP* mRNA expression after oocyte vitrification (Wen *et al.* 2014; Jo *et al.* 2015), using the expression of CIRBP as a marker of cryoinjury. Aside from its roles in hypothermia, CIRBP has been shown to play key roles during early development (Peng *et al.* 2000) and cell protection against endogenous and external stressors at normothermia (Lleonart 2010). Our study only analyzed an early event during oocyte maturation and no later events on development. Future research should focus on the effects of CIRBP and other CIPs during *in vitro* early embryo development and tests its impact on embryo competence after oocyte vitrification/warming.

Another recently described RNA-binding protein induced by mild hypothermia and other stressors is SRSF5 (Fujita *et al.* 2017). SRSF5 belongs to the serine-arginine rich splicing factor (SRSF) protein family, which is involved in alternative splicing and mRNA processing (Änkö 2014). Additionally, SRSF5 seems to perform specific functions in pluripotent cells, managing the nuclear availability of splicing factors and cytoplasmic activities by responding quickly to changing cellular circumstances (Botti *et al.* 2017). Our results showed that mild hypothermia increased *SRSF5* mRNA expression in cumulus cells but not in oocytes. Besides, *SRSF5* mRNA expression was increased by the vitrification procedure in both oocytes and cumulus cells. These results contrast with what was observed in *CIRBP* and *RBM3* mRNA expression, where only mild hypothermia was responsible for most of the changes. Although SRSF5 has been recently described as a novel CIP (Fujita *et al.* 2017), its mRNA expression responded differently from *CIRBP* and *RBM3*.

The use of mild hypothermia sub-lethal stress and the vitrification procedure lead to analyzing apoptotic-related transcripts in this study. BCL2 is an anti-apoptotic gene that promotes cells survival by releasing mitochondrial cytochrome c (Yang *et al.* 1997), whereas BAX is the first pro-apoptotic member activated during apoptotic conditions, accelerating cell death (Yang and Rajamahendran 2002). Other studies have investigated the effect of vitrification on the expression of both genes (García-Martínez *et al.* 2020; Shirzeyli *et al.* 2021); however, the results have been inconsistent. Our results showed that mild hypothermia and vitrification affected *BCL2* and *BAX* mRNA expression. Taken together, the detrimental effects caused by mild hypothermia and vitrification induced apoptotic pathways in oocytes and cumulus cells were independent of the zr17-2 and CIRBP exogenous supplementation. Consistent with this conclusion, our BAX:BCL2 ratio data confirmed this hypothesis.

CIRBP has an important role in regulating inflammatory molecules like IL-1β and the NF-κB pathway (Brochu *et al.* 2013), and extracellularly, CIRBP acts as a damage-associated molecular signal (Aziz *et al.* 2019). Notably, IL-1β is a cytokine that plays a crucial role in inflammation, stimulating its production and other pro-inflammatory cytokines (Dinarello 1996; Eskan *et al.* 2008). Cytokines operate as mediators between the immune and reproductive systems and paracrine and autocrine ovarian regulatory factors (Halme *et al.* 1985; Gorospe and Kasson 1988; Adashi 1990). In addition to ovarian macrophages and monocytes, other ovarian cell types can produce cytokines, like the granulosa cells (Hurwitz *et al.* 1992). Besides, the supplementation with IL-1β and tumor necrosis factor alpha stimulates the growth and maturation of bovine oocytes during *in vitro* culture (Lima *et al.* 2018). Our study showed an upregulation of *IL-1β* mRNA expression in cumulus cells after vitrification. However, our data cannot link the changes in *IL-1β* mRNA expression with the stimulation of the COCs growth after warming. Further research is needed to test if IL-1β could stimulate the COCs growth after warming to cope with the detrimental effects of vitrification. Somehow, increased concentrations of zr17-2 exogenous supplementation trigger the downregulation of *IL-1β* mRNA expression. These results suggest a link between the two molecules, perhaps via a regulatory function of CIRBP accumulation (Brochu *et al.* 2013), but further studies should be performed to define the exact mechanism behind these results.

Conclusions

In conclusion, the current study has identified differences in the mRNA expression of bovine oocytes and cumulus cells exposed to mild hypothermia and vitrification. In addition, the exogenous supplementation with zr17-2 to the maturation media has little impact on the CIPs mRNA expression of both oocytes and cumulus cells, but it affects *IL-1β* mRNA expression under physiological temperatures and at the higher concentration used. Mild hypothermia induced most of the mRNA expression changes observed in this study. CIPs mRNA expression increased in response to mild hypothermia. In addition, *SRSF5* mRNA expression among the CIPs included in this study was affected by vitrification. Vitrification also affected the mRNA expression of apoptotic-related genes and IL-1β. Future studies are needed to assess whether the identified mRNA expression changes might affect the overall oocyte quality and, perhaps, the subsequent embryo development. Understanding how CIPs influence oocyte maturation could help establish new protocols that prompt protection against vitrification-induced damage.

Acknowledgements: we would like to express our gratitude to the employees of the *Escorxador de Sabadell* (Barcelona, Spain) for their help in the ovaries collection.

Declaration of interest: None.

Author contributions: JG collected the samples, conducted the experiments, analyzed and interpreted the data, performed the statistical analyses, and wrote the original draft of the manuscript. MRC and JGS helped with the experiments and collection of the samples. JGS, AM, TM, MLB, and MAR supervised the study. AM, TM, MLB, and MAR were responsible for the funding. All authors revised and approved the final version of the manuscript.

Financial support: This research was funded by the projects PID2019-108320RJ-I00, IJCI-2015-24380, AGL2016-79802-P and PID2020-116531RB-I00. JG was supported by the Government of Catalonia (Generalitat de Catalunya, AGAUR) co-financed with the European Social Found (2018FI_00236). MRC is supported by the Government of Spain, Ministry of Education, Culture and Sports (Training programme for Academic Staff FPU15/06029).

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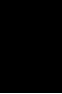
CHAPTER III



The mRNA expression of the three major described cold-inducible proteins, including CIRBP, differs in the bovine endometrium and ampulla during the estrous cycle

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This chapter is under review in *Research in Veterinary Science*.

Abstract

The estrous cycle promotes remodeling of the female reproductive tract induced by hormonal fluctuations. Different factors are involved in maintaining the functions during the estrous cycle. The cold-inducible proteins (CIPs) are essential for post-transcriptional gene regulation playing diverse tissue-specific roles in maintaining normal cellular function and morphogenesis. The potential implications of these genes in reproductive events and fertility raise questions about the role of the CIPs and related factors in the physiology of the bovine reproductive tract. However, the expression changes of CIPs during the bovine estrous cycle have not been studied so far. Here, we hypothesized that the bovine estrous cycle could affect the mRNA expression of the CIPs and other candidate transcripts in the reproductive tract. This study aimed to examine estrous cycle-dependent mRNA expression patterns in the bovine endometrium and ampulla of three of the major described CIPs (*CIRBP*, *RBM3*, *SRSF5*), a set of inflammatory cytokines (*IL-10*, *IL-18*, *IL-1β*), and other candidate genes (*IL-10RA*, *IL-10RB*, *BCL2*, *NLRP3*, *STAT1*, *STAT3*, *STAT5A*, *STAT6*). Endometrial and ampullar tissues were assessed by RT-qPCR. Additionally, the mRNA expression levels were correlated among them and with follicular progesterone and estradiol concentrations. The transcript levels of CIPs increased in the endometrium during stage III (Days 11-17) compared to stage I (Days 1-4) and IV (Days 18-20). In the ampulla, the mRNA expression of *CIRBP* increased during the late luteal phase (stage III), but no differences in the expression of other CIPs were observed. This study expands the current knowledge regarding mRNA expression in the endometrium and oviductal ampulla of cycling heifers, focusing mainly on the CIPs. A better understanding of the mechanisms within the uterus and oviduct during the estrous cycle is crucial to improving fertility rates.

Keywords: cold-inducible RNA-binding protein; qPCR; Western blot; cattle

Introduction

During the bovine estrous cycle, different transcriptomic changes are induced by hormonal regulation along the female reproductive tract in preparation for subsequent reproductive events (Forde *et al.* 2011). Several gene regulatory processes are activated in response to a wide range of stressors to organize an appropriate cellular response (Harvey *et al.* 2017). The post-transcriptional control of RNAs provides a critical regulatory point in gene expression (Änkö 2018). This regulation is achieved, in part, by the RNA-binding proteins (RBPs), which form RNA ribonucleoproteins complexes when binding to RNA, defining the processing, localization, lifetime, and translation rate of specific mRNAs (Lunde *et al.* 2007). The RBPs are involved in several functions, including the modulation of inflammation and the immune system (Kafasla *et al.* 2014), contributing to different reproductive pathophysiological conditions (Khalaj *et al.* 2017). Although inflammation can cause pathological states in the female reproductive tract, an appropriate pro- and anti-inflammatory balance is required for reproductive success (Cicchese *et al.* 2018). Besides, inflammation is crucial during several physiological reproductive steps, including ovulation, mating, embryo implantation (Jabbour *et al.* 2009), and the estrous cycle (Bauersachs *et al.* 2005). However, the precise mechanism by which RBPs are modulated in the reproductive tissues during the bovine estrous cycle is poorly understood.

A family of evolutionarily conserved RBPs named cold-inducible proteins (CIPs) (Ciuzan *et al.* 2015) is transcriptionally upregulated not only in response to mild hypothermia (Danno *et al.* 1997; Nishiyama *et al.* 1997; Tong *et al.* 2013; Rzechorzek *et al.* 2015; Fujita *et al.* 2017), but also to different cellular stressors, such as UV radiation, osmotic stress, or hypoxia (Zhu *et al.* 2016). The cold-inducible RNA-binding protein (CIRBP) is a constitutively and ubiquitously expressed CIP (Liao *et al.* 2017). Other CIPs are the RNA-binding motif protein 3 (RBM3) and the serine and arginine-rich splicing factor 5 (SRSF5), also induced by mild hypothermia and other general stressors (Zhu *et al.* 2016; Fujita *et al.* 2017). CIRBP is induced by mild hypothermia in bovine cumulus cells (Gardela, Ruiz-Conca, *et al.* 2019), and it is localized in the bovine ovary (Gardela, García-Sanmartín, *et al.* 2019) and the endometrium of women (Hamid *et al.* 2003). Several findings suggest that CIPs play various tissue-specific roles, including maintenance of normal cellular function and morphogenesis (Zhu *et al.* 2016). However, if the bovine estrous cycle modulates the mRNA expression of these CIPs along the female reproductive tract has not been determined.

Generally, CIRBP is mainly found in the nucleus but can be translocated to the cytoplasm and released extracellularly under certain stress situations (Aziz *et al.* 2019). Intracellularly, CIRBP acts as an RNA chaperone regulating the mRNA stability of specific targets and transmitting signals interacting with other proteins (Zhong and Huang 2017). Under stress, CIRBP can suppress apoptotic pathways promoting cell survival (Liao *et al.* 2017), enhancing the expression of anti-apoptotic proteins like the B-cell lymphoma 2 (BCL2), which prevents apoptosis by maintaining cell survival (Yang *et al.* 1997).

Extracellular CIRBP functions as a danger-associated molecular pattern (DAMP) response to promote inflammation (Qiang *et al.* 2013), being involved in diverse acute and chronic inflammatory diseases (Zhong and Huang 2017). Besides, CIRBP has been related to the inflammasome (Yang *et al.* 2016). This multiprotein complex signaling platform that mediates immune responses activating inflammatory caspases that facilitate the secretion of cytokines, which induce a pro-inflammatory form of cell death (Broz and Dixit 2016). The protein complex is formed in response to infection, damage, or other cellular stressors, containing a nucleotide-binding oligomerization domain-like receptor (NLR) sensor molecule (Latz *et al.* 2013). The NLR pyrin domain containing 3 (NLRP3) inflammasome is a crucial mediator of inflammation in response to DAMPs, including cell-free DNA, extracellular debris, or extracellular vesicles (Broz and Dixit 2016; Shirasuna *et al.* 2020). The activation of the inflammasome complex triggers caspase 1, which activates the pro-inflammatory interleukins IL-1β and IL-18 (Latz *et al.* 2013). IL-1β mediates immune cell activation, cytokine production, and adhesion molecules, playing essential roles in several inflammatory diseases (Dinarello 1996). IL-18 is a pro-inflammatory cytokine first identified for its interferon- γ -inducing properties (Okamura *et al.* 1998).

As an anti-inflammatory cytokine, IL-10 is secreted by many cell types, playing crucial roles in maintaining maternal immune tolerance (Cheng and Sharma 2015). The biological effects of IL-10 are exerted through binding to the tetramer IL-10 receptor (IL-10R), with two ligand-binding subunits (IL-10R1 or IL-10RA) and two accessory signaling subunits (IL-10R2 or IL-10RB) (Cheng and Sharma 2015). Binding IL-10 to its receptor inhibits autophagy through the phosphoinositide-3K (PI3K)-Akt signaling pathway (Park *et al.* 2011) and activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which could activate STAT3 promoting various IL-10-responsive genes (Donnelly *et al.* 1999). The JAK-STAT pathway regulates the transcription of multiple genes relevant to immunity, development, and metabolic and stress responses (Verhoeven *et al.* 2020). Activated by multiple cytokines (including interferons (INFs) and interleukins) (Bromberg 2001), STATs are involved in several cell processes, and their dysregulation can induce pathological events (Bowman *et al.* 2000).

Revealing the mRNA changes of CIPs and inflammatory and immune-related factors may help better understand the biological processes in the endometrium and ampulla during the bovine estrous cycle. Here, we hypothesized that the mRNA expression of the CIPs is differently present through the bovine estrous cycle stages and in different regions of the female reproductive tract. Taking the ampulla and the endometrium as essential tissues for reproductive success in which fertilization (Ellington 1991) and implantation (Lee and DeMayo 2004) take place, respectively, we aimed to explore the mRNA expression of three major described CIPs (*CIRBP*, *RBM3*, and *SRSF5*), inflammatory interleukins (*IL-10*, *IL-1β*, and *IL-18*), as well as other relevant immunity, cell proliferation, and apoptosis genes (*IL-10RA*, *IL-10RB*, *BCL2*, *NLRP3*, *STAT1*, *STAT3*, *STAT5A*, *STAT6*).

Materials and methods

Animals and tissue collection

Commercial crossbreed beef postpubertal heifers (*Bos taurus*) aged between 13-15 months were used for the recovery of the female reproductive tracts (n = 20). Animals were slaughtered for commercial purposes in a local slaughterhouse (*Escorxador de Sabadell*, Barcelona, Spain). The bovine reproductive tracts were classified into different stages of the estrous cycle by the appearance of corpora lutea and antral follicles according to the morphological classification described by Ireland *et al.* (Ireland *et al.* 1980), as follows: stage I, corresponding to the post-ovulatory phase, Days 1 to 4 (n = 6); stage II, corresponding to the early luteal phase, Days 5 to 10 (not included due to the low number of animals found); stage III, corresponding to the late luteal phase, Days 11 to 17 (n = 8); and stage IV, corresponding to the pre-ovulatory phase, Days 18 to 20 (n = 6). Day 0 was considered as the day on which the female exhibits estrus (standing to be mounted) and other proceptive phase behaviors (vocalization, mounting herd mates, and restlessness) (Estill 2015). Endometrial tissue samples were obtained exposing the endometrial mucosa from intercaruncular areas at the base of the uterine horn ipsilateral to the pre-ovulatory or freshly ovulated follicle, less than 1 h from the harvest of the female reproductive tracts. Additionally, whole sections of the ampulla of the ipsilateral oviduct were obtained. During the RNA extraction process, only mucosal samples were analyzed. Immediately after tissue collection, each sample was immersed in RNA*later*™ stabilization solution (Fisher Scientific, Gothenburg, Sweden) and stored at −80°C until RNA extraction.

Follicular progesterone and estradiol concentrations

Follicular fluid was collected by aspiration of subordinate and dominant follicles present in the ovaries using an 18 G needle coupled with a 10-mL syringe. An individual pool of fluid from subordinate and dominant follicles from each ovary was used to determine follicular concentrations of estradiol and progesterone. Samples were centrifuged at 5,000× *g* for 10 min to discard cellular debris. Supernatants were recovered and stored at -20°C until their analysis.

Follicular progesterone and estradiol concentrations were determined by ELISA (Progesterone ELISA KIT and Estradiol ELISA KIT; Neogen Corporation, Ayr, UK). The absorbance values were obtained using a microplate reader (Sunrise-Basic Tecan; Tecan Austria GmbH, Grödig, Austria). According to the manufacturer, cross-reactivity of the progesterone ELISA antibody with other steroids was: deoxycorticosterone 2.5%, corticosterone 2.0%, and pregnenolone 2.0%. Steroids with a cross-reactivity <2.0% are not presented. The cross-reactivity of the estradiol ELISA antibody with other steroids was testosterone 1.0%. Steroids with a cross-reactivity <1.0% are not presented. The precision within the test was assessed by calculating coefficients of variation (CV, where CV = SD/mean × 100) from all duplicate samples. The CV for progesterone and estradiol were 7.47% and 3.65%, respectively. A sensitivity of 0.23 ng/mL and 0.012 ng/mL for progesterone and estradiol were respectively obtained. Finally, for each pair of progesterone and estradiol data, a progesterone/estradiol (P4/E2) ratio was calculated.

RNA extraction and cDNA synthesis

Samples were homogenized in TRIzol using a bead mill (24 beats/s, 2 min, twice) (TissueLyser II with 7 mm stainless steel beads; Qiagen, Sollentuna, Sweden) for total RNA extraction, as reported (Gardela, Jauregi-Miguel, *et al.* 2020). The homogenized was centrifuged (12,000× *g*, 10 min, 4°C) and the supernatant was retrieved and mixed with bromochloropropane (100 µL/mL homogenized). After centrifugation (12,000× *g*, 15 min, 4°C), the aqueous phase was incubated with isopropanol and RNA precipitation solution (1.2 M NaCl and 0.8 M $Na_2C_6H_6O_7$) (250 µL of each/500 µL aqueous phase). The mixtures were centrifuged (12,000× *g*, 10 min, 4°C) and the supernatants were discarded. For each sample, 1 mL 75% ethanol was added. Supernatants were discarded after centrifugation (7,500× *g*, 5 min, 4°C), and the RNA pellets obtained were dried for 30 min in the fume hood. The RNA was dissolved in 30 µL of RNase-free water for 30 min on ice.

After the extraction, the RNA concentration was determined using the Thermo Scientific NanoDrop™ 2000 (Fisher Scientific, Gothenburg, Sweden). The quality of the RNA was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), using the samples with an RNA integrity number (RIN) value higher than 8. The cDNA synthesis was

performed using the High-Capacity RNA-to-cDNA™ Kit (Fisher Scientific, Gothenburg, Sweden) according to the manufacturer's instructions. The reaction consisted of 5 µg RNA in a final volume of 50 µL. The cDNA obtained was stored at -20°C for subsequent analysis.

Quantitative real-time PCR analyses

Following a previous protocol for quantitative real-time polymerase chain reaction (qPCR) (Gardela, Jauregi-Miguel, *et al.* 2020) (CFX96™; Bio-Rad Laboratories, Inc; Kabelsketal, Germany), the mRNA relative expression levels were quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The reactions consisted of 2 µL cDNA, 250 nM of each primer, and 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA) in a final volume of 10 µL. The PCR protocol consisted of one cycle of uracil-DNA glycosylase activation at 50°C for 2 min; one cycle of denaturation at 95°C for 2 min; and 40 cycles of denaturation at 95°C for 5 s, annealing/extension at 60.2°C for 30 s, and a melting curve at 60-95°C (0.5 increments) for 5 s/step. Two technical replicates were performed per each sample and primer pair used. Four housekeeping genes were initially used for cDNA normalization (G3DPH, β-ACTIN, HPRT1, and TBP). After a preliminary analysis of the results, the G3PDH was the most constantly expressed gene through the tissues and treatments and was chosen for further analyses. Commercial gene-specific qPCR primers for bovine were used (PrimePCR™SYBR® Green Assay, Bio-Rad Laboratories, Kabelsketal, Germany). The specific sequences for each primer are registered by the company. The product sizes for each primer are shown in Table **1S** and were confirmed by loading the amplicons in an agarose gel using a gel imaging system (ChemiDoc XRS+ System, BioRad Laboratories, Inc., Kabelsketal, Germany).

Protein extraction and Western blot analyses

Tissue fragments were homogenized in radioimmunoprecipitation assay lysis buffer (RIPA; Fisher Scientific, Gothenburg, Sweden) supplemented with 10 µL/mL protease inhibitors and ethylenediaminetetraacetic acid (Thermo Scientific™ Halt™ Protease Inhibitor Cocktail (100×); Fisher Scientific, Gothenburg, Sweden). Homogenates were centrifuged (13,000× *g*, 10 min, 4°C) and the supernatants were collected. Protein concentration was determined by the detergent compatible (DC) protein assay (Bio-Rad Laboratories, Inc; Kabelsketal, Germany), using bovine serum albumin as standard. Then, 25 µg of each sample were mixed with 4× sample buffer (NuPAGE LDS; Fisher Scientific, Gothenburg, Sweden) and 500 mM dithiothreitol. Samples were heated for 10 min at 70°C. Samples were run on 12-15% SDS-polyacrylamide gels. Odyssey® One-Color Protein Molecular Weight Marker (LI-COR Biosciences, Inc; Hamburg, The Netherlands) was used as a molecular weight marker. For Western blot analysis, proteins were transferred onto 0.2 µm polyvinylidene difluoride (PVDF) membranes (Fisher Scientific,

Gothenburg, Sweden), previously activated with methanol. For protein identification, and after blocking (Intercept Tris-buffered saline blocking buffer; LI-COR Biosciences, Inc; Hamburg, The Netherlands) for 1 h at room temperature, membranes were incubated overnight at 4°C with a rabbit monoclonal antibody against CIRBP (ab191885, Abcam, Cambridge, UK) at dilution 1:500. To standardize the results, a rabbit monoclonal antibody against α-tubulin (ab52866, Abcam, Cambridge, UK) was used at dilution 1:1,000 in the same membranes. To visualize immunoreactivity, membranes were incubated with secondary antibody donkey anti-rabbit IgGs (IRDye® 800 CW-labelled, 926-32213, LI-COR Biosciences, Inc; Hamburg, The Netherlands). Then, PVDF membranes were scanned with Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, Inc; Hamburg, The Netherlands). Optical density was quantified by Image Studio software version 5.2. (LI-COR Biosciences, Inc; Hamburg, The Netherlands).

Statistical analyses

Data from qPCR analyses were exported with CFX Maestro™ 1.1 software version 4.1.2433.1219 (Bio-Rad Laboratories, Inc; Kabelsketal, Germany) and data from Western blot analyses were exported with Image Studio software version 5.2. (LI-COR Biosciences, Inc; Hamburg, The Netherlands). All data sets were analyzed for normal distribution and homoscedasticity using the Shapiro–Wilk Normality test and Levene's test, respectively. The log(x+1) was used to transform non-normal distributions before analysis to achieve a normal distribution. Statistical analyses were performed with R software version 3.6.1. (R Core Team 2019). Data are presented as mean ± SEM. The threshold for significance was set at $p < 0.05$.

Data from qPCR, Western blot, and follicular hormone analyses were analyzed using the *nlme* package (Pinheiro *et al.* 2020) to develop linear mixed-effects (LME) models and the *multcomp* package (Hothorn *et al.* 2008) to perform pairwise comparisons adjusted by Tukey's test. In the LME model, the estrous cycle stages were included as the fixed factor and the samples as the random part of the model. Data from qPCR analyses were also analyzed using multiple Spearman's rank correlation coefficients to explore the relationship between the mRNA expression fold change of the target genes and between mRNA expression and follicular fluid hormone concentrations. Data from Western blot analyses were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. Additionally, to investigate whether the CIRBP protein abundance occurs in proportion to its mRNA relative levels, Spearman's rank correlation coefficients were performed.

Results

Endometrial mRNA expression changes

The analysis indicated that the differences observed in the endometrium between stages of the estrous cycle in the mRNA expression of *CIRBP*, *RBM3*, *SRSF5*, *STAT1*, *STAT5A*, *STAT6*, *IL-10RB*, and *NLRP3* were statistically significant (Figure **1**). The mRNA expression of the CIPs (*CIRBP*, *RBM3*, and *SRSF5*) was higher expressed in stage III of the estrus cycle compared to stage I and IV ($p < 0.05$). Similarly, *STAT1* and *STAT6* mRNA expressions were higher expressed in stage III of the estrous cycle compared to stage I and IV ($p < 0.05$). The *STAT5A* and *NLRP3* mRNA expressions were higher expressed in stages I and IV of the estrous cycle compared to stage III ($p < 0.05$). The *IL-10RB* mRNA expression was higher expressed in stage III of the estrus cycle compared to stage I of the estrus cycle ($p < 0.05$).

Ampullary mRNA expression changes

The analysis showed statistical differences in *CIRBP*, *STAT1*, *STAT3*, *STAT5A*, *STAT6*, *BCL2*, *IL-10RA*, *IL-10RB*, and *IL-1β* mRNA expression between stages of the estrous cycle in the ampulla (Figure **2**). Of the three CIPs included in the study, only differences in the mRNA expression of CIRBP were found, increasing its mRNA expression in stage III of the estrus cycle compared to stage I and IV ($p < 0.05$). Similarly, *STAT5A*, *IL-10RA*, and *IL-10RB* mRNA expressions were higher expressed in stage III of the estrous cycle compared to stage I and IV ($p < 0.05$). Conversely, *BCL2*, *STAT3*, and *IL-1β* mRNA expression were lower expressed in stage III of the estrous cycle compared to stage I and IV ($p < 0.05$). The *STAT1* and *STAT6* mRNA expressions were higher expressed in stage I of the estrous cycle compared to stage III ($p < 0.05$).

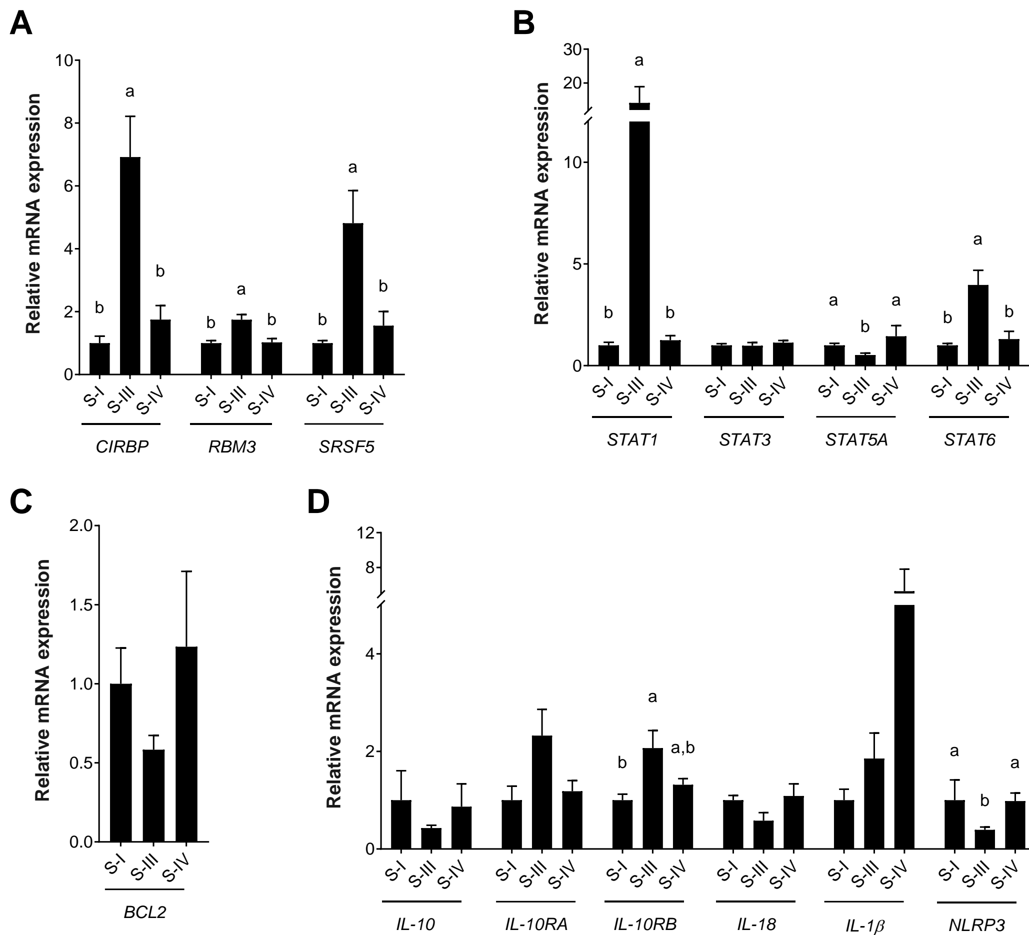

Figure 1. Endometrial relative mRNA expressions changes of the genes included in the study. Genes involved in (**A**) cold-inducible proteins, (**B**) factors of the signal transducer and activator of transcription (STAT) family, (**C**) the anti-apoptotic gene *BCL2*, and (**D**) inflammation, comparing stage I (S-I, n = 6), stage III (S-III, n = 8), and stage IV (S-IV, n = 6) of the bovine estrus cycle. Stage I was established as the reference group. Different letters show statistical differences between the bovine estrus cycle stages for the same gene ($p < 0.05$). Mean ± SEM.

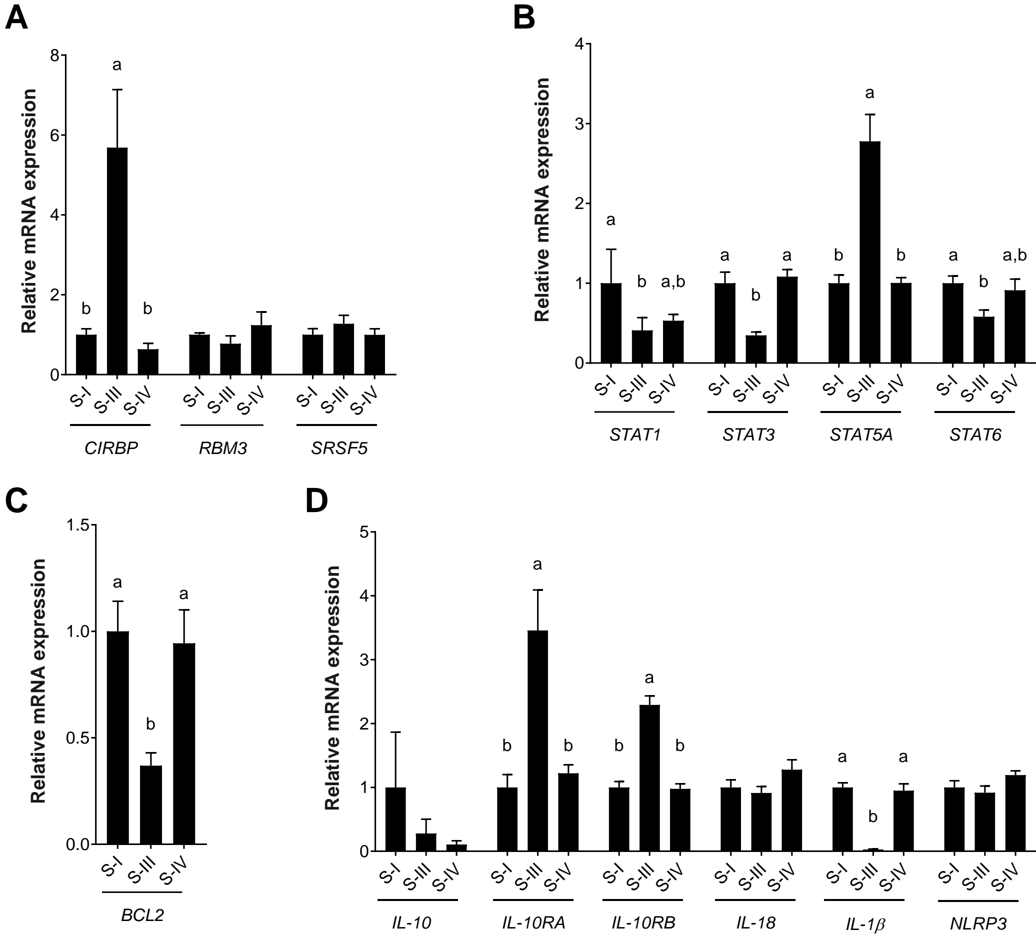

Figure 2. Ampullary relative mRNA expressions changes of the genes included in the study. Genes involved in (**A**) cold-inducible proteins, (**B**) factors of the signal transducer and activator of transcription (STAT) family, (**C**) the anti-apoptotic gene *BCL2*, and (**D**) inflammation, comparing stage I (S-I, n = 6), stage III (S-III, n = 8), and stage IV (S-IV, n = 6) of the bovine estrus cycle. Stage I was established as the reference group. Different letters show statistical differences between the bovine estrus cycle stages for the same gene (p < 0.05). Mean ± SEM.

Correlations between relative mRNA expression of genes and follicular hormone concentrations

The follicular fluid progesterone and estradiol concentrations depicted a non-significant increasing trend as the estrous cycle progresses (Figure **3A**). The progesterone/estradiol (P4/E2) ratio did not differ significantly between the stages of the estrous cycle (Figure **3B**).

The relative mRNA expression levels of the genes included in the study were correlated among themselves and with the follicular fluid concentrations for both tissues analyzed, namely the endometrium (Figure **1S**) and the ampulla (Figure **2S**). The endometrial *CIRBP* mRNA expression positively correlates with several mRNAs, independently of the stage of the estrous cycle. On the other hand, the ampullary *CIRBP* mRNA expression negatively correlates with interleukins and their receptors, and with *NLRP3* during stage III.

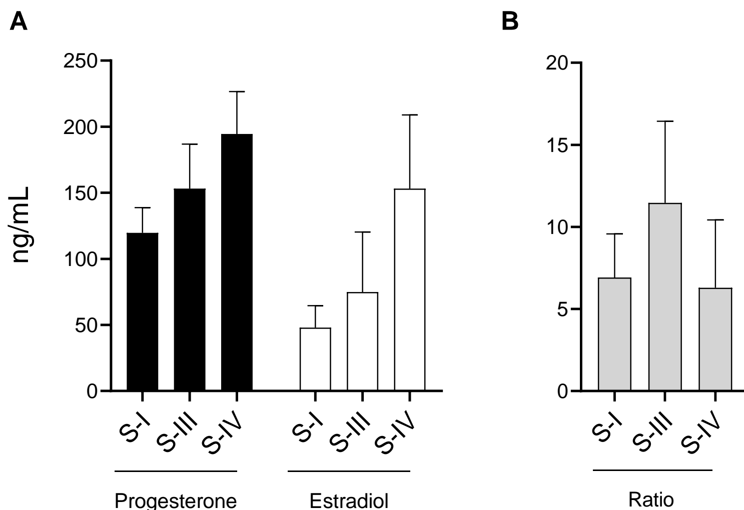

Figure 3. Follicular fluid hormone concentrations and ratio progesterone/estradiol. (**A**) Follicular fluid progesterone and estradiol concentrations (ng/mL) and (**B**) ratio progesterone/estradiol during stage I (S-I, n = 6), stage III (S-III, n = 8), and stage IV (S-IV, n = 6) of the bovine estrus cycle. Non-significant differences between stages were found. Mean ± SEM.

CIRBP protein expression in the bovine endometrium and ampulla

Differences in relative CIRBP protein expression between stages of the estrous cycle were evidenced by Western blot analysis of both in the endometrial and ampullary tissues (Figure 4). In the endometrium, the CIRBP protein expression was higher expressed in stage III compared to stage I ($p < 0.05$). In the ampulla, the CIRBP protein expression was higher expressed in stages I and III compared to stage IV ($p < 0.05$).

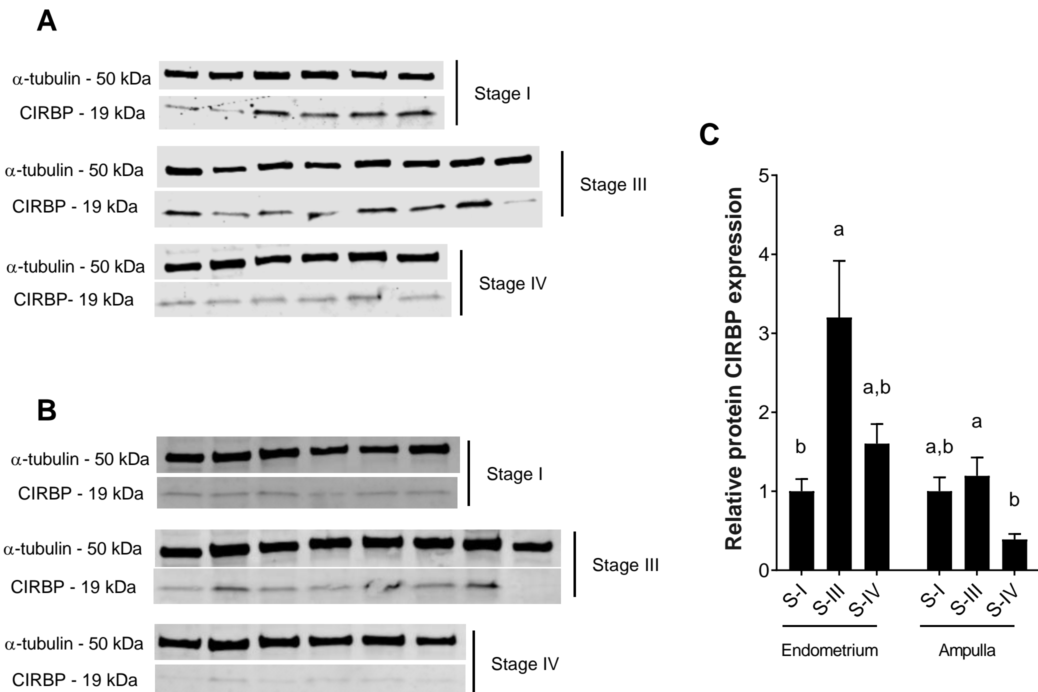

Figure 4. Relative CIRBP protein expression in endometrium and ampulla. Western blot analyses of relative CIRBP protein expression in (**A**) the endometrium and (**B**) ampulla. (**C**) Relative protein expression of CIRBP in the endometrium and the ampulla, comparing stage I (S-I, n = 6), stage III (S-III, n = 8), and stage IV (S-IV, n = 6) of the estrus cycle. Stage I was established as the reference group. Different letters show statistical differences between the estrus cycle stages for each tissue ($p < 0.05$). Mean ± SEM.

CIRBP protein abundance in proportion to CIRBP mRNA levels

In both endometrial and ampullary tissues, the CIRBP protein expression correlated with the abundances of its corresponding mRNA, but not strongly (Figure **5**). The Spearman correlation coefficient of 0.61 in the endometrium implied that 61% of the variation in CIRBP protein expression was explained by known mRNA abundances. Similarly, the Spearman correlation coefficient of 0.56 in the ampulla implied that 56% of the variation in CIRBP protein expression was explained by known mRNA abundances.

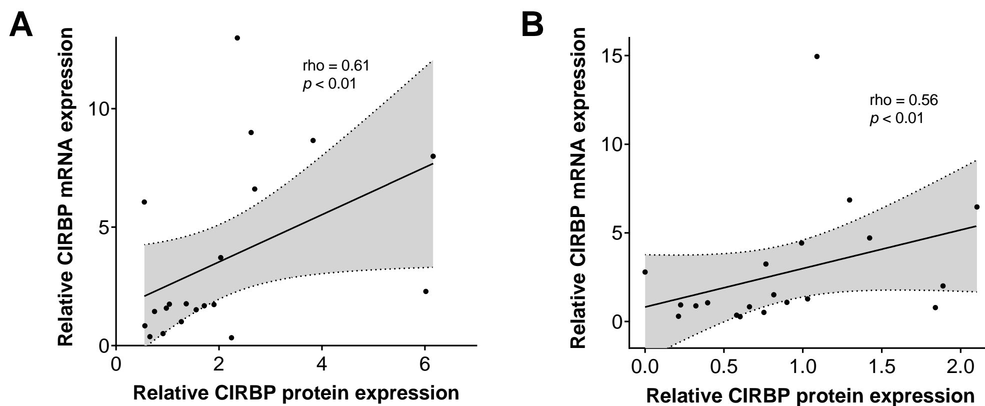

Figure 5. Correlation between relative CIRBP mRNA and protein expression. Spearman rank correlation scatter plots and linear regression with its 95% confidence interval (gray area) of the relative CIRBP mRNA expression and the CIRBP protein expression (**A**) in the endometrium and (**B**) ampulla.

Discussion

The RBPs have key functions in the post-transcriptional regulation of mRNA, being crucial in tissue homeostasis and pathophysiology in multiple tissues, including the female reproductive tract (Khalaj *et al.* 2017; Änkö 2018). Although the implications of RBPs in the reproductive process are poorly understood, CIPs may have potential implications in early reproductive events during the peri-ovulatory phase (Gardela, Ruiz-Conca, *et al.* 2020). The present study demonstrates that the mRNA of CIPs and other candidate factors are differentially expressed in the endometrium and ampulla during the bovine estrous cycle.

It has been hypothesized that the regular cyclic changes induced during the estrous cycle in the bovine female reproductive tract may lead to changes in the mRNA expression of three CIPs: *CIRBP*, *RBM3*, and *SRSF5*. However, as multifunctional proteins that contribute to the

maintenance of normal cellular function (Zhu *et al.* 2016), we aimed to explore other candidate-related factors. Our results showed increased the CIRBP, RBM3, and SRSF5 mRNA expression in the endometrium during the late luteal phase (stage III). Only *CIRBP* relative mRNA level was increased in the ampulla during stage III of the estrous cycle, which was also confirmed by results obtained at the protein level. This situation resembles that reported for the human uterus, whose CIRBP protein levels in the endometrium are strongly increased during the secretory (luteal) phase and inversely correlated to the proliferative activity in endometrial glands (Hamid *et al.* 2003). These results may be related to the described activities of CIRBP in the regulation of post-transcriptional and translational events, cell proliferation, and protection against apoptosis, among other molecular and cellular activities (Zhu *et al.* 2016). However, this matter needs further research to be clarified.

High progesterone levels in the luteal phase can induce apoptotic-like regression of granulosa cells (Zhang *et al.* 2021). However, apoptosis is less important in the oviduct changes during the estrous cycle (Steffl *et al.* 2008). As shown here, we demonstrate differential expression of the anti-apoptotic gene *BCL2* and its correlations with other genes during the bovine estrous cycle in the ampulla. The mRNA expression of *BCL2* increased during the pre- and post-ovulatory phases (stage IV and I, respectively), showing a common positive correlation with the mRNA expression of *CIRBP*, *SRSF5*, *IL-1β*, and *IL-18*, and a negative correlation with the mRNA expression of *IL-10*. Our data agree with the proposed defense mechanism to protect the ampulla from damage caused by apoptosis during the luteal phase (Zhang *et al.* 2021).

Il-1β stimulates the infiltration and migration of immune cells to the bovine oviduct epithelium (Nakamura *et al.* 2021). In the present study, the mRNA expression of *IL-1β* was significantly higher in the ampulla during pre-ovulatory and post-ovulatory phases compared with the late luteal phase. Similar results were reported in the bovine endometrium, in which the mRNA expression of *IL-1β* increased around ovulation compared to the luteal phase (Fischer *et al.* 2010). Other studies demonstrate the induction of inflammatory cytokines and acute-phase proteins in the bovine endometrium during post-partum (Gabler *et al.* 2010; Pothmann *et al.* 2021), first inflammatory immune responses (Gärtner *et al.* 2016), and endometritis (Wagener *et al.* 2017). However, our data did not show *IL-1β* mRNA expression changes in the endometrium, probably due to the variation of mRNA expression between animals. As previously proposed in the bovine endometrium by Ficher and collaborators (2010), we suggest a similar functional IL1 system in the bovine ampulla.

Our results did not show any changes in *IL-10* mRNA expression either in the endometrium or ampulla during the bovine estrous cycle. However, we demonstrated higher *IL-10RA* and *IL-10RB* mRNA expression in the ampulla during the late luteal phase, and higher *IL-10RB* mRNA

expression in the endometrium during the late luteal phase that may suggest a role of these receptors in the homeostasis maintenance in that specific stage of the estrus cycle.

Several immune-mediated processes contribute to the regulation of reproductive physiology (Yockey and Iwasaki 2018) and may be beneficial for subsequent fertility at the time of insemination (Wagener *et al.* 2021). Our results revealed higher *NLRP3* mRNA expression in the endometrium during the pre- and post-ovulatory phases. As long as the NLRP3 has been linked with the preparation of the bovine endometrium during early implantation (Suzuki *et al.* 2018), our results might suggest that, in the absence of pregnancy, the increase of *NLRP3* mRNA expression could lead to the preparation of the endometrium for the early reproductive events.

We analyzed the factors of the STAT family that can activate gene transcription, transducing signals from the cell membrane into the nucleus (Sadowski *et al.* 1993). Here, we reported differential and opposite *STAT1*, *STAT3*, *STAT5A*, and *STAT6* mRNA expressions during the bovine estrous cycle in the endometrium and ampulla.

Our results showed an increase in the endometrium *STAT1* mRNA expression during the late luteal phase, being the STAT1 one of the first STAT proteins detected in the INF signal transduction pathways, which involves, among others, stimulation of apoptosis (Bromberg 2001). Previous studies have shown that, in the event of gestation, the *STAT1* transcript increases in the endometrium during the period of maternal recognition of pregnancy (Day 16) and then amplifies at implantation (Day 20) (Carvalho *et al.* 2016). In the ampulla, the *STAT1* mRNA expression was increased during the post-ovulatory phase compared to the late luteal phase. This dual modulation of *STAT1* mRNA opens new questions to understand the biological functions and specific mechanism of STAT1 during the bovine estrous cycle.

The activation of STAT3, by multiple cytokines or other factors, such as hormones (Verhoeven *et al.* 2020), is crucial for wound healing (Chang *et al.* 2004), restoration of tissue integrity (Sano *et al.* 1999), and the resolution of the immune response (Fielding *et al.* 2008). Our results showed a decrease in *STAT3* transcript in the ampulla during the late luteal phase, suggesting that STAT3 signaling in the ampulla may be involved in early reproductive events, like ovulation, sperm transport, or fertilization. As for the STAT6, previous studies have associated STAT6 with puberty onset (Nguyen *et al.* 2018) and age at first corpus luteum (Fortes *et al.* 2011). As shown here, *STAT6* mRNA expression increased in the ampulla during the pre- and post-ovulatory phases, contrary to what happened the endometrium. These results may suggest possible roles of STAT6 during early reproductive events, yet not fully understood.

Our study gives a first approach to understanding the modulation of the CIPs and related transcripts during the bovine estrous cycle. Regarding the classification of the reproductive tracts, the methodology applied has been used for over 40 years to assign animals into stages and the

length of such stages; however, the lack of complete analysis to confirm that classification should be considered, and the presence of animals in different hormonal profiles and physiological statuses should not be discarded. Moreover, further studies are needed to link the different correlation patterns between follicular hormones and mRNA expression levels observed during the bovine estrous cycle in this study.

Conclusions

In summary, we have revealed significant changes in the mRNA expression during the bovine estrous cycle. Here, we reported an increase in the mRNA and protein expression of *CIRBP* during the late luteal phase (Days 11-17) in both the endometrium and ampulla of cycling heifers. Additionally, we demonstrated changes in the mRNA expression of other CIPs (*RBM3* and *SRSF5*), a pro-inflammatory cytokine (*IL-1β*), and other related genes involved in inflammatory pathways (*IL-10RA*, *IL-10RB*, *BCL2*, *NLRP3*, *STAT1*, *STAT3*, *STAT5A*, *STAT6*). Our data provide novel molecular insights into the mRNA changes during the bovine estrous cycle and raises questions about the regulation and functions of CIPs under non-hypothermic physiological situations, but further validation is needed for its application as prospective biomarkers for reproductive physiology performance.

Ethics approval: Not applicable.

Declaration of interest: None.

Author contributions: JG: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing – original Draft, Visualization; MRC: Methodology, Investigation, Writing – Review & Editing; SOM: Methodology, Validation, Investigation, Writing – Review & Editing; MLB: Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding acquisition, MAR: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Acknowledgements: The authors are grateful for the staff from the *Escorxador de Sabadell* slaughterhouse (Sabadell, Barcelona, Spain), and for the infrastructure support from Linköping University, including genomics core facility. We also thank Amaia Jauregi Miguel, for her kind assistance with the qPCR procedure and validation.

Funding: This research was funded by the Grant PID2019-108320RJ-I00 and IJCI-2015-24380 funded by MCIN/ AEI /10.13039/501100011033 (Spain) and FEDER funds (EU). JG was supported by the Government of Catalonia (Generalitat de Catalunya, AGAUR) co-financed with the European Social Found (2018FI_00236) and was awarded with an international mobility grant from Col·legi Oficial de Veterinaris de Barcelona (COVB, Beques Ramon Turró). MRC is supported by the Government of Spain, Ministry of Education, Culture and Sports (Training programme for Academic Staff FPU15/06029) and Ministry of Universities (Mobility grant for short stays and temporary transfers for beneficiaries of the FPU program; EST19/00848).

Table 1S. Primers used for the quantitative real-time PCR analyses.

Gene	Product size (bp)	Genebank accession number	Melting temperature
CIRBP	170	XM_005209265, XM_005209263, XM_005209264, XM_005209266, NM_001034278, XR_235246, XR_235245, XR_235247	87.5°C
BCL2	96	NM_001166486, XM_005224107, XM_005224106, XM_005224105, XM_005224108	86.0°C
G3PDH	92	N/A	85.5°C
IL-10	120	NM_174088	85.5°C
IL-10RA	99	XM_005215831, NM_001205757	86.0°C
IL-10RB	71	NM_001076975	82.0°C
IL-18	77	XM_005215801, NM_174091	77.0°C
IL-1β	111	XM_005212737, NM_174093	84.0°C
NLRP3	100	XM_005209231, NM_001102219	82.5°C
RBM3	61	N/A	80.0°C
SRSF5	100	N/A	82.0°C
STAT1	89	XM_005202571, XM_005202570, XM_005202574, XM_005202573, XM_005202572, NM_001077900	80.5°C
STAT3	114	XM_005220691, XM_005220692, NM_001012671, XM_005220693	84.5°C
STAT5A	65	NM_001012673	83.5°C
STAT6	65	XM_005206514, XM_005206515, NM_001205501	79.5°C

CIRBP: cold-inducible RNA-binding protein; *BCL2*: B-cell lymphoma 2; *G3PDH*: glyceraldehyde 3-phosphate dehydrogenase; *IL-10*: interleukin 10; *IL10RA*: interleukin 10 receptor, α subunit; *IL10RB*: interleukin 10 receptor, β subunit; *IL-18*: interleukin 18; *IL-1β*: interleukin 1 β; *NLRP3*: NLR family pyrin domain containing 3; *RBM3*: RNA-binding motif protein 3; *SRSF5*: serine and arginine rich splicing factor 5; *STAT1*: signal transducer and activator of transcription 1; *STAT3*: signal transducer and activator of transcription 3; *STAT5A*: signal transducer and activator of transcription 5A; *STAT6*: signal transducer and activator of transcription 6; bp: base pair; N/A: not available.

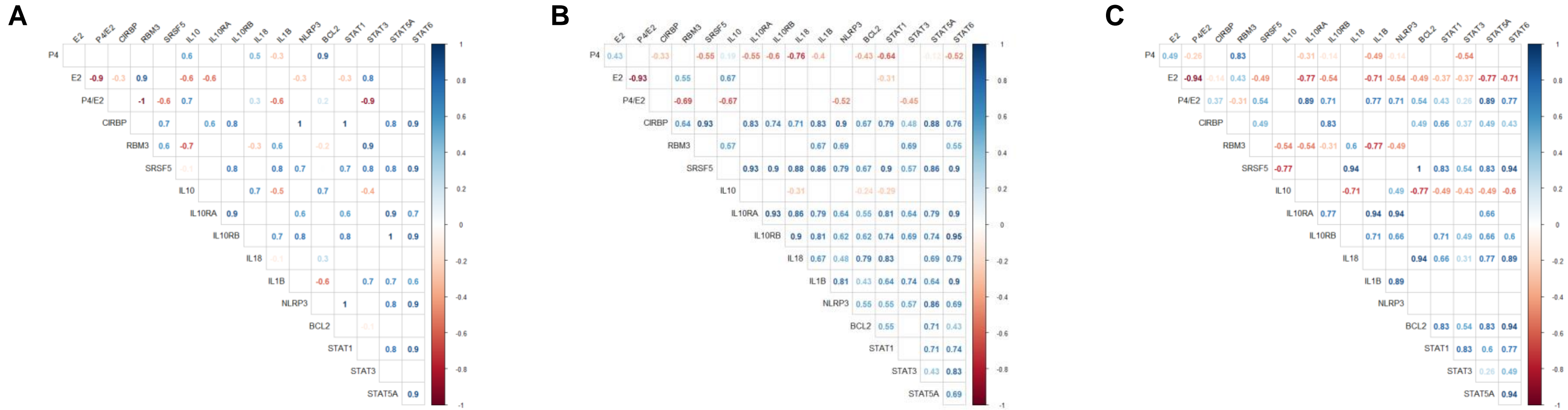

Figure 1S. Endometrial Spearman rank correlations of the mRNA expression of the genes included in the study. Correlations during (**A**) stage I, (**B**) stage III, and (**C**) stage IV of the bovine estrous cycle. Significant positive correlation coefficients are displayed in blue ($p < 0.05$) and significant negative correlation coefficients are displayed in red ($p < 0.05$). Non-significant correlation coefficients are omitted.

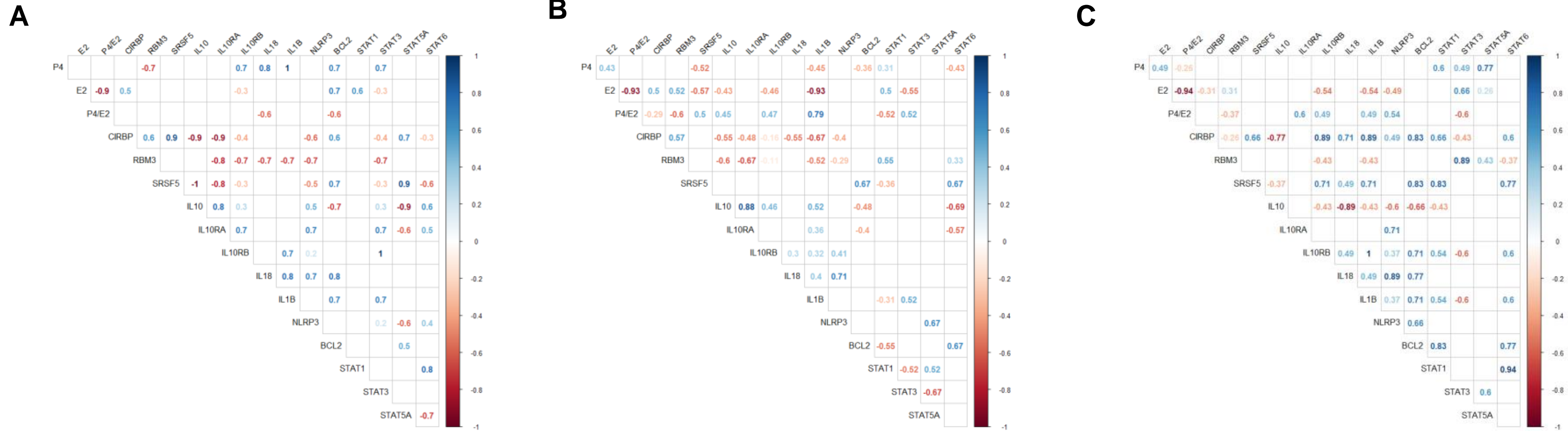

Figure 2S. Ampullary Spearman rank correlations of the mRNA expression of the genes included in the study. Correlations during (**A**) stage I, (**B**) stage III, and (**C**) stage IV of the bovine estrous cycle. Significant positive correlation coefficients are displayed in blue ($p < 0.05$) and significant negative correlation coefficients are displayed in red ($p < 0.05$). Non-significant correlation coefficients are omitted.

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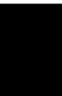
CHAPTER IV



The expression of cold-inducible RNA-binding protein mRNA in sow genital tract is modulated by natural mating, but not by seminal plasma

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This chapter has been published in *International Journal of Molecular Sciences.*

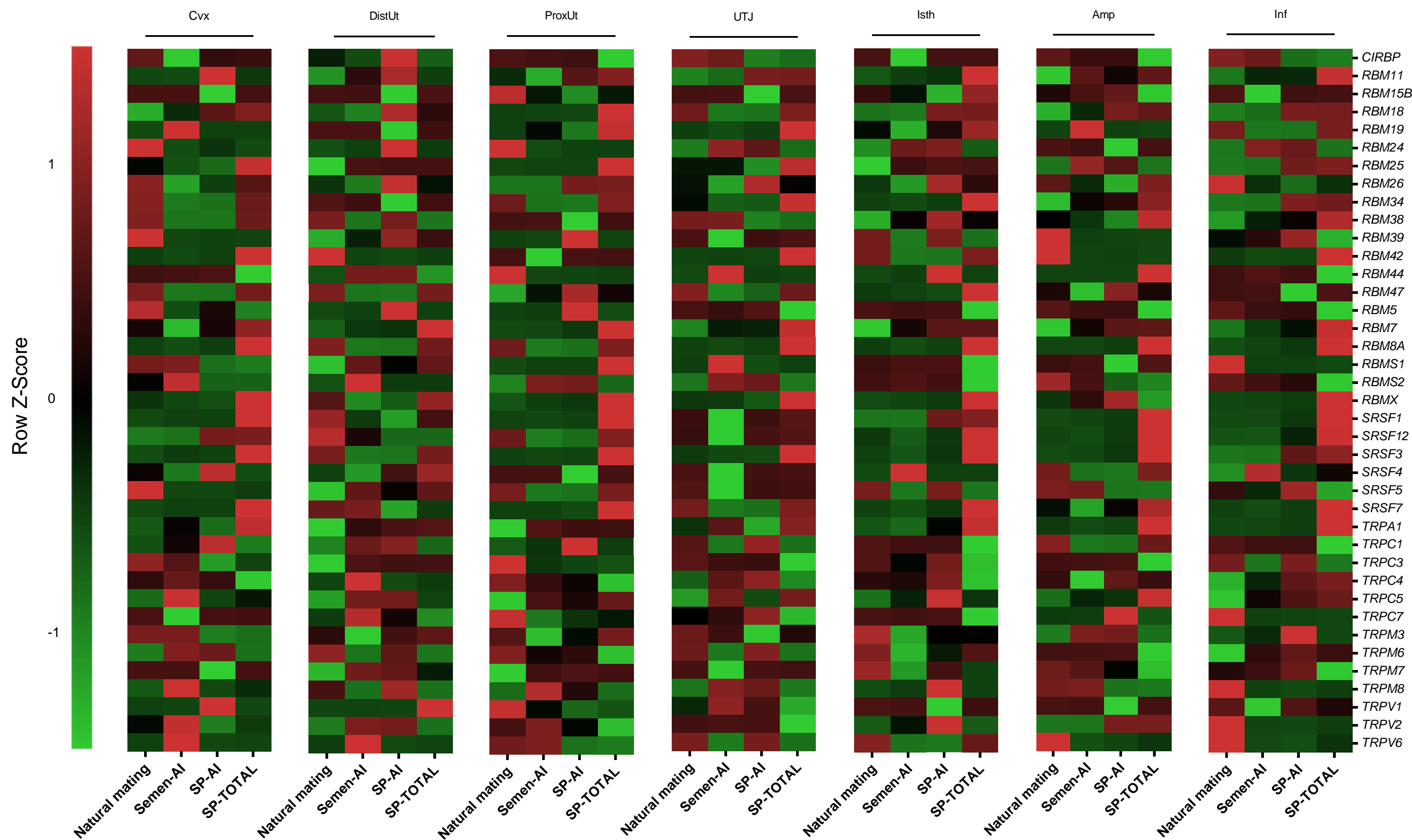
(Int. J. Mol. Sci. 2020;21(15):5333. doi: 10.3390/ijms21155333)

Abstract

The RNA-binding proteins (RBPs), some of them induced by transient receptor potential (TRP) ion channels, are crucial regulators of RNA function that can contribute to reproductive pathogenesis, including inflammation and immune dysfunction. This study aimed to reveal the influence of spermatozoa, seminal plasma, or natural mating on mRNA expression of RBPs and TRP ion channels in different segments of the internal genital tract of oestrous, preovulatory sows. Particularly, we focused on mRNA expression changes of the cold-inducible proteins (CIPs) and related TRP channels. Pre-ovulatory sows were naturally mated (NM) or cervically infused with semen (Semen-AI) or sperm-free seminal plasma either from the entire ejaculate (SP-TOTAL) or the sperm-rich fraction (SP-AI). Samples (cervix to infundibulum) were collected by laparotomy under general anesthesia for transcriptomic analysis (GeneChip® Porcine Gene 1.0 ST Array) 24 h after treatments. The NM treatment induced most of the mRNA expression changes, compared to Semen-AI, SP-AI, and SP-TOTAL treatments including unique significative changes in *CIRBP*, *RBM11*, *RBM15B*, *RBMS1*, *TRPC1*, *TRPC4*, *TRPC7*, and *TRPM8*. The findings on the differential mRNA expression on RBPs and TRP ion channels, especially to CIPs and related TRP ion channels, suggest that spermatozoa and seminal plasma differentially modulated both protein families during the preovulatory phase, probably related to a still unknown early signaling mechanism in the sow reproductive tract.

Keywords: transcriptomics; microarrays; spermatozoa; seminal plasma; cold-inducible proteins; cold-sensitive TRP ion channels; pig

Graphical Abstract



Introduction

Mammals reproduce by internal fertilization releasing billions of spermatozoa into the female reproductive tract to achieve maximum chances of fertilization, despite only thousands reaching the oviduct (Rodríguez-Martínez *et al.* 2005), where fertilization takes place. Ejaculate deposition into the female reproductive tract affects the molecular and cellular function of reproductive organs proximal and distal to the insemination site (Parada-Bustamante *et al.* 2016). The interaction between the female reproductive tract and semen induces mRNA and protein expression changes in endometrial and oviductal tissues modulating important processes for reproduction, such as angiogenesis, sperm storage and selection, oviduct contractility and oocyte transportation, and preimplantation embryo development, previously reported in pigs (Georgiou *et al.* 2007; Krawczynski and Kaczmarek 2012; Almiñana *et al.* 2014; López-Úbeda *et al.* 2015) and other species (Chan *et al.* 2001; Orihuela *et al.* 2001; Bauersachs *et al.* 2003; Fazeli *et al.* 2004; Artemenko *et al.* 2015).

Many RNA-binding proteins (RBPs) are involved in the control of inflammation and immune dysfunction, which can contribute to pathogenesis of reproductive disorders (Khalaj *et al.* 2017). Each step of RNA metabolism (transcription, splicing, polyadenylation, stabilization, edition, capping, or translation) is assisted by the RBPs (Bock *et al.* 2015). These proteins are the most important regulators of RNAs and crucial for gene regulation, containing different structural RNA-binding motifs or domains, such as RNA recognition motif (RRM), K homology domain, double-stranded RNA binding domain, or zinc fingers, forming ribonucleoprotein complexes by binding double or single-stranded RNA (Lunde *et al.* 2007). Beyond their RNA-binding domains, the RBPs hold many structural modules that facilitate protein-protein interactions and catalytic events; thereby, they are incorporated in numerous intracellular processes (Lunde *et al.* 2007).

The cold-inducible proteins (CIPs) are evolutionarily conserved RBPs that have been detected in multiple organisms from different taxa (Ciuzan *et al.* 2015). The cold-inducible RNA-binding protein (CIRBP, also known as CIRP, or heterogenous nuclear ribonucleoprotein A18, hnRNP A18), the RNA-binding motif protein 3 (RBM3), and the serine and arginine-rich splicing factor 5 (SRSF5, also known as SRp40) are CIPs transcriptionally up-regulated in response to moderately low temperatures and other cellular stressors, such as hypoxia, DNA damage, or osmotic stress (Zhu *et al.* 2016; Fujita *et al.* 2017).

CIRBP was first described in the late 1990s as a novel RBP which expression was induced in mouse cells in response to cold stress (Nishiyama *et al.* 1997). Since this discovery, investigations revealed its role in many cellular stress responses, such as mRNA stability, cell survival, proliferation, stress adaptation, and tumor formation (Aziz *et al.* 2019). Intracellularly, CIRBP acts as a RNA chaperone to facilitate translation, whereas extracellularly, it seems to play a crucial role in the promotion of inflammation and injury (Qiang *et al.* 2013; Rajayer *et al.* 2013).

Both CIRBP and RBM3 are structurally similar to the heterogeneous nuclear ribonucleoproteins and belong to the glycine-rich RBP family class IVa (Ciuzan *et al.* 2015), which contains, at the N-terminal end, one conserved RRM and two ribonucleoprotein domains (RNPs), known as RNP1 and RNP2 (Zhu *et al.* 2016). The C-terminal contains a less conserved arginine-glycine-rich (RGG) domain, and this is the reason why CIRBP and RBM3 belong to the large family of glycine-rich proteins (Zhu *et al.* 2016). SRSF5 belongs to the serine/arginine-rich family protein (also called serine/arginine-rich splicing factors, SRSFs), characterized by the presence of one or two N-terminal RRMs followed by a downstream domain rich in arginine and serine residues (Manley and Krainer 2010). Heterogeneous nuclear ribonucleoproteins and SRSFs are both active factors involved in the regulation of pre-mRNA splicing, and due to its derived origin from a common ancestor, have been suggested to play an important role in the evolution of the alternative splicing mechanism (Busch and Hertel 2012). Through alternative splicing, a single gene can encode for multiple variants, greatly enhancing the transcriptome complexity, the variety of proteins and hence, the diverse biological functions (Black 2003).

The induction of CIPs is dependent on transient receptor potential (TRP) V4, V3, and M8 ion channel proteins (Fujita *et al.* 2017; Fujita *et al.* 2018). The TRP mammalian proteins can be classified in four major families (Nilius and Voets 2005): the ankyrin-related TRP channel family (TRPA), the TRPC family (classical or canonical), the melastatin-related TRP channel family (TRPM), and the channels homologous to vanilloid receptor (TRPV). These receptors are non-selective cation channels permeable for Ca^{2+} that open in response to multiple chemical and physical stimuli (Damann *et al.* 2008; Holzer and Izzo 2014; Uchida *et al.* 2017), providing Ca2+ influx pathways (Nilius and Voets 2005). Most of them have thermosensitive abilities, like TRPM8, TRPA1, and TRPC5, which have been proposed as cold-sensitive ion channels (Uchida *et al.* 2017).

Even though current research has focused on discerning the effects produced by spermatozoa and seminal plasma (SP) in endometrium and oviductal mRNA expression in the sow reproductive tract (Alvarez-Rodriguez *et al.* 2019; Álvarez-Rodríguez *et al.* 2020; Martinez *et al.* 2020), to the best of our knowledge, there is a lack of literature regarding the differential mRNA expression levels of genes encoding for RBPs and TRP ion channels. Here, we specifically studied the differential mRNA expression levels of CIPs and related TRP ion channels in the internal genital

tract of pre-ovulatory sows induced by the interaction of semen (spermatozoa and/or SP) with the genital mucosa.

Results

The presence of inflammation was analyzed by the mRNA expression of the interleukin-8, also called CXCL8, a chemokine induced in lipopolysaccharide-stimulated monocytes inducing neutrophil migration (Russo *et al.* 2014) and often used as classical inflammation marker. In this study, mRNA expression of CXCL8 was down-regulated ($p < 0.05$) by natural mating (NM) in the cervix (fold change −4.38) and the proximal uterus (fold change −3.06), and down-regulated ($p < 0.05$) by artificial insemination with the sperm-peak fraction (Semen-AI) in the utero-tubal junction (fold change −5.78), whereas no differences were found in the rest of the tissues and treatments. Spermatozoa were only present in the utero-tubal junction of NM and Semen-AI treated animals but not in animals treated with sperm-free SP or controls.

Induced mRNA Expression Changes on RBPs and TRP Ion Channels

Table **1** displays the mRNA expression levels of the 39 differentially expressed genes (DEGs) encoding for RBPs and TRP ion channels for each treatment and tissue sample. The number of DEGs (up- and down-regulated, $p < 0.05$), with respect to the control group (cervical infusion with 50 mL Beltsville thawing solution, BTS), that codify for RBP family containing the RRM domain (IPR000504) were displayed in Figure **1a**. Even though the statistical analyses did not determine overall differences among groups in the number of up-regulated DEGs encoding for RBPs (NM: 16 genes, Semen-AI: 6 genes, sperm-free SP infusion from the sperm-peak fraction (SP-AI): 8 genes, and sperm-free SP infusion from the whole ejaculate (SP-TOTAL): 9 genes; $p > 0.05$), the infundibulum showed a greater number of up-regulated DEGs encoding for RBPs (12 genes, $p < 0.05$) compared to the proximal uterus (2 genes), within all treatments. Sperm-containing treatments down-regulated a greater number of DEGs encoding for RBPs (NM: 24 genes, and Semen-AI: 21 genes; $p < 0.01$) compared to sperm-free SP treatments (SP-AI: 3 genes, and SP-TOTAL: 1 gene).

Figure **1b** displays the number of DEGs (up- and down-regulated, $p < 0.05$) that codify for TRP ion channels. A greater number of DEGs encoding for TRP ion channels were up-regulated by NM (9 genes, $p < 0.05$) compared to SP-TOTAL (0 genes). The down-regulated DEGs encoding for TRP ion channels were significantly affected by NM (14 genes, $p < 0.001$) compared to the rest of the treatments (Semen-AI: 4 genes, SP-AI: 1 gene, and SP-TOTAL: 2 genes).

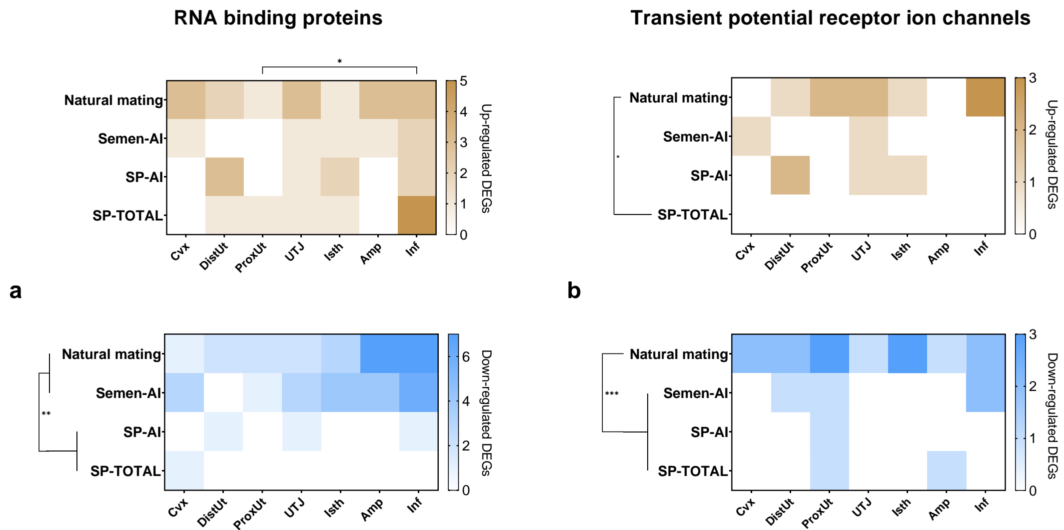


Figure 1. Heatmap composition of the number of differentially expressed genes ($p < 0.05$) in the porcine female reproductive tract 24 h post-treatment. Genes belonging to: (**a**) the RNA-binding protein family that contain the RNA recognition motif domain (IPR000504) in their structure, and (**b**) the transient receptor potential ion channels, up-regulated (brown) and down-regulated (blue). Cvx: cervix; DistUt: distal uterus; ProxUt: proximal uterus; UTJ: utero-tubal junction, Isth: isthmus; Amp: ampulla; Inf: infundibulum. Natural mating: sows mated with a boar; Semen-AI: sows artificially inseminated with the sperm-peak portion extended to 50 mL with Beltsville thawing solution (BTS); SP-AI: sows cervically infused with the sperm-free seminal plasma (SP) from pooled sperm-peak portion (50 mL); SP-TOTAL: sows cervically infused with the sperm-free SP of the whole ejaculate (50 mL). All treatments were compared with controls (cervical infusion with 50 mL BTS). Asterisks represent statistical differences between treatments and tissues (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The number of common DEGs that codify for RBPs and TRP ion channels (up- and down-regulated, $p < 0.05$) in the reproductive tract of sows between treatments were displayed in a series of Venn diagrams (Heberle *et al.* 2015), indicating which DEGs were identified as common to each treatment per tissue (Figure 2). As may be seen, the mRNA expression pattern was affected by the entrance of the early sperm-rich fraction of the ejaculate in the female reproductive tract (Figure **2a**). The common DEGs between NM an Semen-AI groups tended to be down-regulated (12 down-regulated DEGs vs. 4 up-regulated DEGs, Figure **2a**). The combination between NM and SP-TOTAL groups only demonstrated common down-regulated DEGs in the uterus (Figure **2b**). Combinations including sperm-free SP treatments showed common up- and down-regulated DEGs generally observed in the uterus (Figure **2b–d**). However, the combination between Semen-AI and SP-AI groups showed common up- and down-regulated DEGs in the

Table 1. Differential mRNA expression ($p < 0.05$) encoding for RNA-binding proteins and transient receptor potential ion channels in the different segments of the sow reproductive tract (cervix to infundibulum), 24 h post-treatment.

Gene Symbol	Natural Mating		Semen-AI		SP-AI		SP-TOTAL	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
*CIRBP**	Cvx (1.62) UTJ (1.27) Amp (1.41) Inf (1.25)	-	-	-	-	-	-	-
RBM11		**Amp (−1.91)** **Inf (−1.73)**	-	-	-	-	-	-
RBM15B	ProxUt (1.19)	-	-	-	-	-	-	-
RBM18	-	**Cvx (−1.38)** **Amp (−1.28)** **Inf (−1.26)**	-	**Cvx (−1.31)**	-	-	-	**Cvx (−1.22)**
RBM19	-	-	-	-	-	**DistUt (−1.15)**	ProxUt (1.18) Isth (1.24)	-
RBM24	-	**Isth (−1.76)**	-	-	Isth (1.22)	-	-	-
RBM25	-	-	-	-	-	-	Inf (1.25)	-
RBM26	-	-	-	-	DistUt (1.11)	-	-	-
RBM34	-	**Amp (−1.53)** **Inf (−1.23)**	-	**Inf (−1.18)**	Inf (1.19)	-	UTJ (1.49)	-
RBM38	Cvx (1.27)	-	-	-	-	**UTJ (−1.33)**	-	-
RBM39	-	-	-	-	DistUt (1.2)	-	-	-
RBM42	-	-	-	**Inf (−1.17)**	-	**Inf (−1.16)**	-	-
RBM44 *LOC100627002*	-	**UTJ (−1.2)**	UTJ (1.39)	-	-	-	-	-

Table 1. Continued.

Gene Symbol	Natural Mating		Semen-AI		SP-AI		SP-TOTAL	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
RBM47	DistUt (1.18)			**UTJ (−1.39)**	-	-	-	-
RBM5	Cvx (1.21) UTJ (1.17) Isth (1.18) Amp (1.21) Inf (1.52)	-	Inf (1.21)	-	UTJ (1.24) Isth (1.15)	-	-	-
RBM7	-	**DistUt (−1.4** **ProxUt (−1.35)** **UTJ (−2)** **Isth (−2.48)** **Amp (−1.98)** **Inf (−2.09)**	-	**Cvx (−1.41), ProxUt (−1.33), Inf (−1.5)**	-	-	-	-
RBM8A	-	**Inf (−1.35)**	-	**Isth (−1.23)** **Inf (−1.25)**	-	-	-	-
RBMS1; LOC102159432	-	**DistUt (−1.23)**	-	-	-	-	-	-
RBMS2	Amp (1.71) Inf (1.74)	**ProxUt (−1.41)**	Cvx (1.41) Isth (1.3) Amp (1.5) Inf (1.4)	-	-	-	-	-
RBMX	-	**Isth (−1.18)**	-	-	-	-	Inf (1.33)	-
SRSF1	-	**Amp (−1.36)**	-	**Amp (−1.31)**	-	-	Inf (1.43)	-

Table 1. Continued.

Gene Symbol	Natural Mating		Semen-AI		SP-AI		SP-TOTAL	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
SRSF12; LOC100739399	DistUt (1.25)	**Amp (−1.46)** **Inf (−1.66)**	-	**Isth (−1.6)** **Amp (−1.53)** **Inf (−1.68)**	-	-	-	-
SRSF3; LOC100523801; LOC106508591	-	**Amp (−1.51)** **Inf (−1.27)**	-	**UTJ (−1.26)** **Isth (−1.28)** **Amp (−1.48)** **Inf (−1.24)**	-	--	Inf (1.5)	-
SRSF4	-	-	-	-	DistUt (1.14)		DistUt (1.16)	-
*SRSF5; LOC100623241 **	UTJ (1.28)	-	-	**Cvx (−1.21)**	Inf (1.19)	-	-	-
SRSF7	-	-	-	**UTJ (−1.25)** **Isth (−1.26)** **Amp (−1.38)**	-	-	Inf (1.61)	-
*TRPA1; LOC100627895 **	-	**Cvx (−2.46)** **DistUt (−7.73)** **ProxUt (−6.34)** **Isth (−1.14)**	-	**DistUt (−2.47)**	-	-	-	-
TRPC1	Inf (1.52)	**ProxUt (−1.43)**	-	-	-	-	-	-
TRPC3	UTJ (1.71)	-	UTJ (1.65)		UTJ (1.64)	-	-	-
TRPC4	ProxUt (2.23) Inf (1.29)	-	-	-	-	-	-	-
*TRPC5 **	-	**Cvx (−2.29)** **DistUt (−2.44)** **ProxUt (−3.31)** **UTJ (−2.65)**	-	**ProxUt (−1.57)**	-	**ProxUt (−1.86)**	-	**ProxUt (−1.39)**

Table 1. Continued.

Gene Symbol	Natural Mating		Semen-AI		SP-AI		SP-TOTAL	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
		Isth (−1.78) **Amp (-1.84)** **Inf (−3.21)**						
TRPC7	Inf (1.1)	-	-	-	-	-	-	-
TRPM3	UTJ (1.74) Isth (3.37)	-	-	-	-	-	-	**Amp (−1.68)**
TRPM6	DistUt (2.13) ProxUt (2.2)	**Inf (−1.25)**	-	-	DistUt (1.55)	-	-	-
TRPM7	-	-		-	DistUt (1.24) Isth (1.12)	-	-	-
*TRPM8; LOC100627434 **	-	**Isth (−1.23)**		-	-	-	-	-
TRPV1	-	-		**Inf (−1.38)**	-	-	-	-
TRPV2	-	-		**Inf (−1.05)**	-	-	-	-
TRPV6	-	-	Cvx (2.32)	-	-	-	-	-

Cvx: cervix; DistUt: distal uterus; ProxUt: proximal uterus; UTJ: utero-tubal junction; Isth: isthmus; Amp: ampulla; and Inf: infundibulum. UP: up-regulation and DOWN: down-regulation (in bold). The fold change for each treatment and tissue is presented between brackets. Asterisks mark genes that codify for RNA-binding proteins included in the cold-inducible proteins and genes that codify for cold-sensitive transient receptor potential ion channels. Natural mating: sows mated with a boar; Semen-AI: sows artificially inseminated with the sperm-peak portion extended to 50 mL with Beltsville thawing solution (BTS); SP-AI: sows cervically infused with the sperm-free seminal plasma (SP) from pooled sperm-peak portion (50 mL); SP-TOTAL: sows cervically infused with the sperm-free SP of the whole ejaculate (50 mL). All treatments were compared with controls.

oviduct (Figure **2c**). The combination between NM and Semen-AI groups showed a predominant number of up- and down-regulated DEGs in the oviductal segments compared to the uterine segments (11 DEGs in oviductal tissues *vs.* 5 DEGs in uterine segments, Figure **2a**). Curiously, the proximal uterus showed a common down-regulation of TRPC5 expression in all combinations. Additionally, the utero-tubal junction showed a common up-regulation of TRPC3 between NM and Semen-AI groups, and SP-AI and Semen-AI groups (Figure **2a,c**).

The number of common DEGs that codify for RBPs and TRP ion channels (up- and down-regulated, $p < 0.05$) in the reproductive tract of sows between treatments were displayed in a series of Venn diagrams (Heberle *et al.* 2015), indicating which DEGs were identified as common to each treatment per tissue (Figure **2**). As may be seen, the mRNA expression pattern was affected by the entrance of the early sperm-rich fraction of the ejaculate in the female reproductive tract (Figure **2a**). The common DEGs between NM and Semen-AI groups tended to be down-regulated (12 down-regulated DEGs vs. 4 up-regulated DEGs, Figure **2a**). The combination between NM and SP-TOTAL groups only demonstrated common down-regulated DEGs in the uterus (Figure **2b**). Combinations including sperm-free SP treatments showed common up- and down-regulated DEGs generally observed in the uterus (Figure **2b–d**). However, the combination between Semen-AI and SP-AI groups showed common up- and down-regulated DEGs in the oviduct (Figure **2c**). The combination between NM and Semen-AI groups showed a predominant number of up- and down-regulated DEGs in the oviductal segments compared to the uterine segments (11 DEGs in oviductal tissues *vs.* 5 DEGs in uterine segments, Figure **2a**). Curiously, the proximal uterus showed a common down-regulation of TRPC5 expression in all combinations. Additionally, the utero-tubal junction showed a common up-regulation of TRPC3 between NM and Semen-AI groups, and SP-AI and Semen-AI groups (Figure **2a,c**).

mRNA Expression Changes on CIPs and Related TRP Ion Channels

Of the total number of DEGs encoding for RBP family included in the study (26 genes, Table **1**), only two genes belong to the CIPs: *CIRBP* and *SRSF5* (Zhu *et al.* 2016; Fujita *et al.* 2017). The NM group up-regulated *CIRBP* mRNA expression in the cervix, utero-tubal junction, ampulla, and infundibulum ($p < 0.05$), and up-regulated *SRSF5* mRNA expression in the utero-tubal junction ($p < 0.05$), whereas Semen-AI down-regulated *SRSF5* mRNA expression in the cervix ($p < 0.05$) and SP-AI up-regulated *SRSF5* mRNA expression in the infundibulum ($p < 0.05$).

Of the total number of DEGs encoding for TRP ion channels included in the study (13 genes, Table **1**), only three genes encode for the cold-sensitive TRP ion channels: *TRPA1*, *TRPC5*, and *TRPM8* (Uchida *et al.* 2017). The NM group down-regulated *TRPC5* mRNA expression in all tissues ($p < 0.05$). As previously stated, all treatments down-regulated *TRPC5* mRNA expression

in the proximal uterus ($p < 0.05$). Besides, NM down-regulated *TRPA1* mRNA expression in the uterus and isthmus ($p < 0.05$), and down-regulated *TRPM8* mRNA expression in the isthmus ($p < 0.05$). Additionally, Semen-AI down-regulated *TRPA1* mRNA expression in the distal uterus ($p < 0.05$).

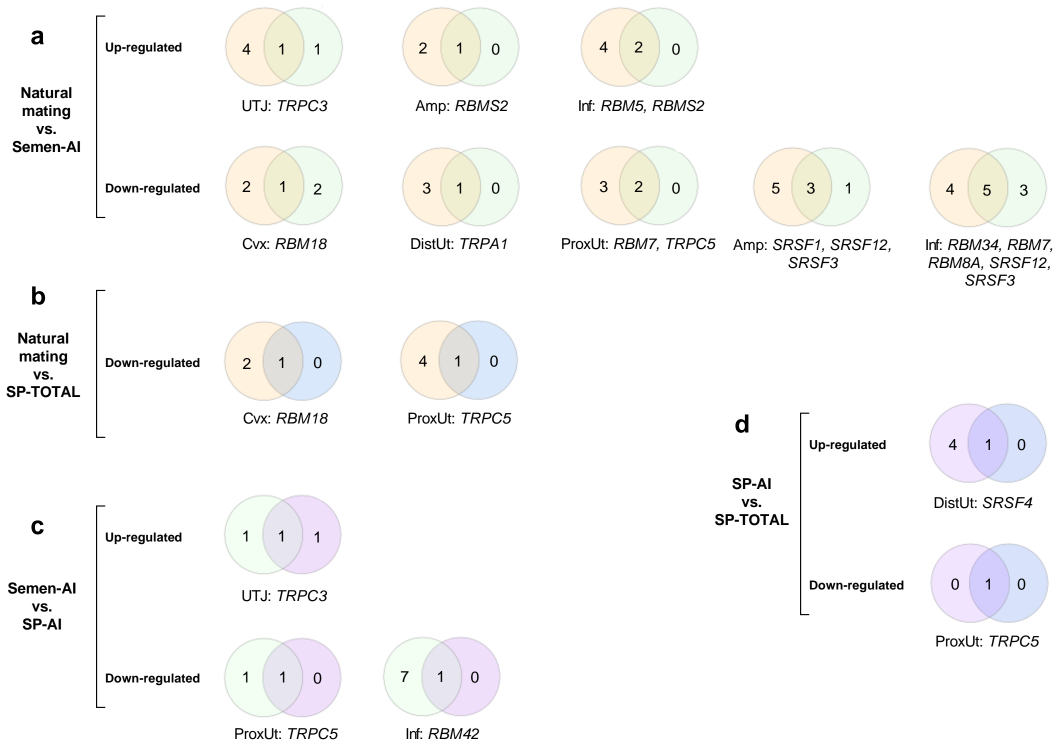


Figure 2. Venn diagrams of commonly differentially expressed genes ($p < 0.05$) in the porcine female reproductive tract codifying for RNA-binding proteins and transient receptor potential ion channels (up- or down-regulated), 24 h post-treatment. (**a**) Sows mated with a boar (Natural mating, orange) or sows artificially inseminated with the sperm-peak portion extended to 50 mL with Beltsville thawing solution (BTS) (Semen-AI, green); (**b**) Natural mating (orange) or sows cervically infused with the sperm-free SP of the whole ejaculate (50 mL) (SP-TOTAL, blue); (**c**) Semen-AI (green) sows cervically infused with the sperm-free seminal plasma (SP) from pooled sperm-peak portion (50 mL) (SP-AI, purple); (**d**) SP-AI (purple) or SP-TOTAL (blue). Cvx: cervix, ProxUt: proximal uterus, DistUt: distal uterus, UTJ: utero-tubal junction, Amp: ampulla, and Inf: infundibulum. The acronyms of genes common to treatment per tissue (crossing sectors) are identified.

Three genes encoding for TRP ion channels are involved in the induction of CIPs: *TRPM8*, *TRPV3*, and *TRPV4* (Fujita *et al.* 2017; Fujita *et al.* 2018). No statistical differences were found for *TRPV3* and *TRPV4* mRNA expression.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analyses

To categorize the function of the 39 DEGs detected, genes were mapped to terms in Gene Ontology (GO) database (Thomas *et al.* 2003) (Figure **3**). Out of the two main categories of the GO classification, the "biological process" was dominant. The predominant molecular function in the sperm-containing treatments was binding activity (GO:0005488) in both up- and down-regulated genes ($p < 0.01$). The predominant biological processes of the down-regulated genes in the sperm-containing treatments were biological regulation (GO:0065007) and cellular process (GO:0009987) followed by metabolic process (GO:0008152) ($p < 0.01$). The predominant biological process in the genes up-regulated by NM was cellular process (GO:0009987), followed by biological regulation (GO:0065007) ($p < 0.05$). No statistical differences were found within molecular functions and biological processes in the sperm-free SP treatments ($p > 0.05$). Molecular functions only differed in tissues where genes were up-regulated by the NM treatment (Figure **3a**) predominantly in the infundibulum compared to endocervix ($p < 0.05$). The biological processes of the genes up-regulated by NM (Figure **3a**) were mostly in the infundibulum and utero-tubal junction compared to the rest of the tissues ($p < 0.05$), whereas the biological processes of the genes down-regulated by NM (Figure **3a**) were dominant in the ampulla and infundibulum compared to the endocervix and isthmus ($p < 0.05$). Similarly, the biological processes of the genes up-regulated by Semen-AI (Figure **3b**) were dominant in the utero-tubal junction compared to the rest of the tissues ($p < 0.001$), whereas the biological processes of the genes down-regulated by Semen-AI (Figure **3b**) dominated in the isthmus, ampulla, and infundibulum compared to the distal uterus ($p < 0.05$). The biological processes of the SP-AI group (Figure **3c**) only differed among up-regulated genes, these being dominant in the distal uterus and utero-tubal junction compared to the endocervix, proximal uterus, isthmus, and ampulla ($p < 0.05$). Conversely, the biological processes of the genes up-regulated by SP-TOTAL (Figure **3d**) appeared mostly in the infundibulum compared to the endocervix, distal uterus, utero-tubal junction, and ampulla ($p < 0.05$), whereas the genes down-regulated by SP-TOTAL (Figure **3d**) dominated the proximal uterus compared to the rest of the tissues ($p < 0.01$).

Altered transcripts were mapped to in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto 2000) and were associated with GO biological process subgroups (Figure **4**). Results showed that the predominant GO biological processes were response to temperature stimulus and RNA transport followed by transmembrane transport, whereas the predominant KEGG pathway was inflammatory mediator regulation of TRP channels followed by mineral absorption. Furthermore, genes were classified by functionally grouped terms and pathways (Figure **5**). The enrichment p-values were predominant in establishment of RNA localization and response to temperature stimulus followed by response to cold, RNA stabilization, and ribonucleoprotein complex assembly.

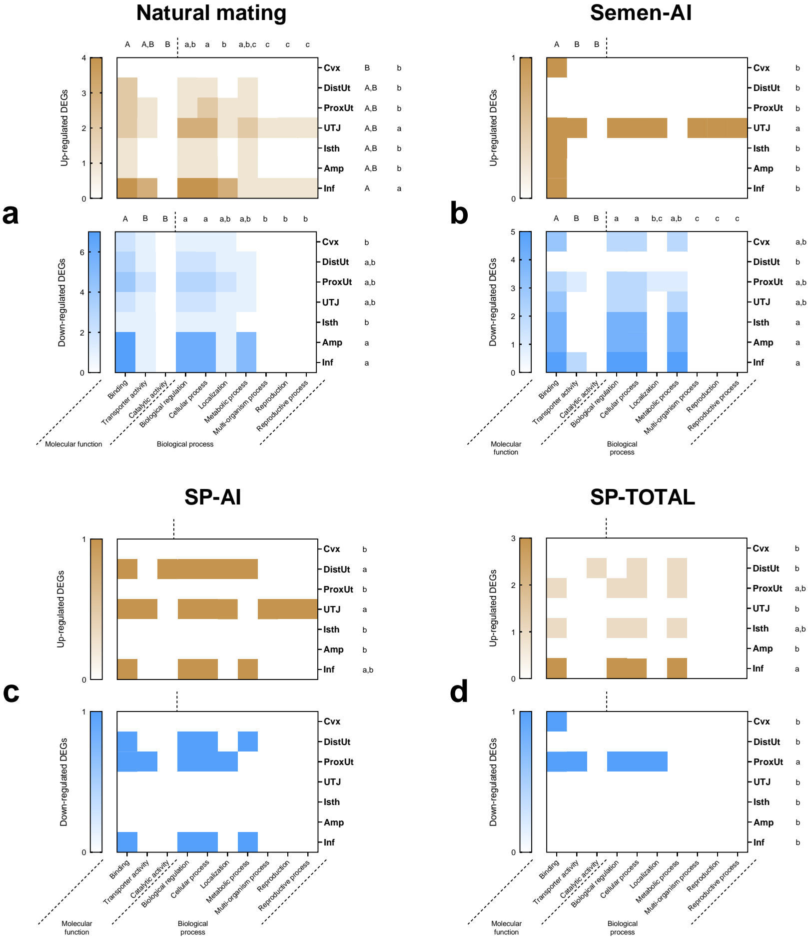


Figure 3. Heatmap composition of Gene Ontology (GO) classification. Categories of the differentially expressed genes ($p < 0.05$), up-regulated (brown) and down-regulated (blue), based on GO function along the segments of the internal female genital tract (Cvx: cervix; DistUt: distal uterus; ProxUt: proximal uterus; UTJ: utero-tubal junction, Isth: isthmus; Amp: ampulla; Inf: infundibulum) after the different treatments: (**a**) Natural mating (sows mated with a boar), (**b**) Semen-AI (sows artificially inseminated with the sperm-peak portion extended to 50 mL with Beltsville thawing solution (BTS)), (**c**) SP-AI (sows cervically infused with the sperm-free SP from pooled sperm-peak portion (50 mL)), and (**d**) SP-TOTAL (sows cervically infused with the sperm-free seminal plasma (SP) of the whole ejaculate (50 mL)). The results are summarized in two major GO categories: molecular function and biological process. Uppercase letters represent differences between molecular function GO categories ($p < 0.05$) and lowercase letters represent differences between biological process GO categories ($p < 0.05$). Differences between tissues in molecular function GO categories are represented in uppercase letters ($p < 0.05$) and differences between tissues in biological process GO categories are represented with lowercase letters ($p < 0.05$).

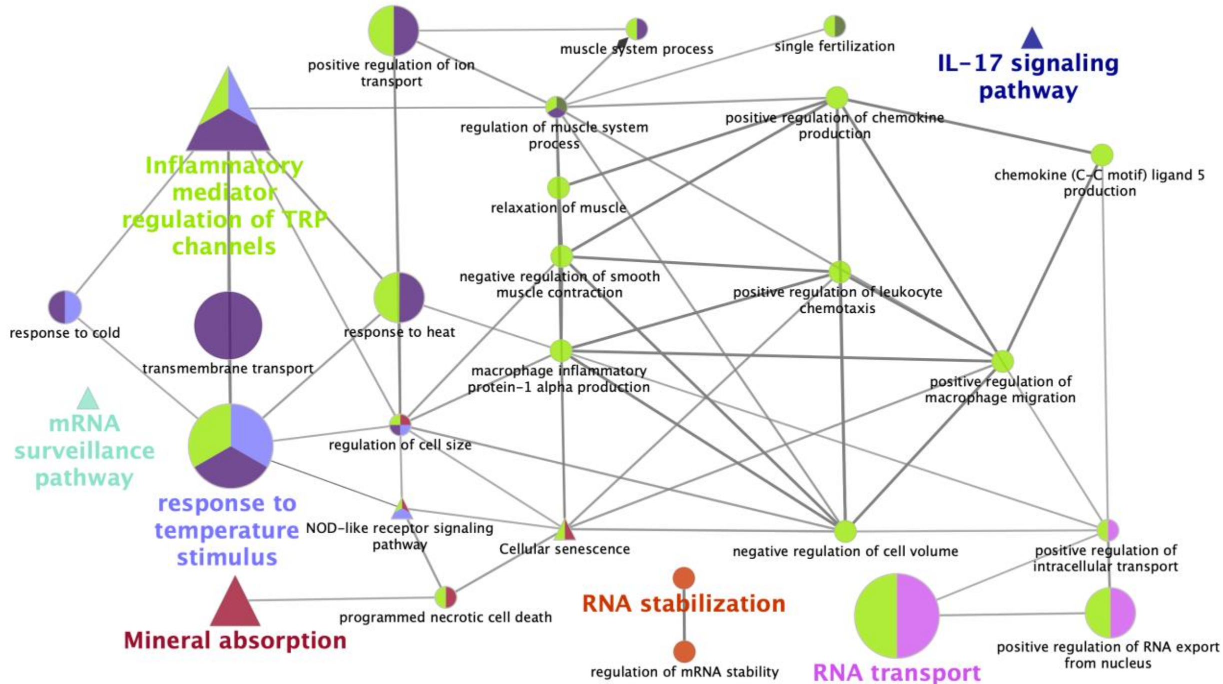


Figure 4. Schematic representation of selected altered transcripts among all tissues and treatments. The analysis of over-represented functional categories was performed using the Cytoscape v3.0.0 application ClueGo v2.0.3. The following databases were used: Gene Ontology (GO) subgroups biological process which is shown as circles and Kyoto Encyclopedia of Genes and Genomes pathways which is shown as triangles. Terms are functionally grouped based on shared genes (kappa score) and are shown in different colours. The size of the nodes indicates the degree of significance, where the biggest nodes correspond to highest significance. The most significant term defines the name of the group. The following ClueGo parameters were used: biological process database (BP; date: 28.03.2019); GO tree levels, 2–6 (first level = 0); minimum number of genes, 3; minimum percentage of genes, 4; 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 was modified; that is, some redundant and noninformative terms were deleted and the network manually rearranged.

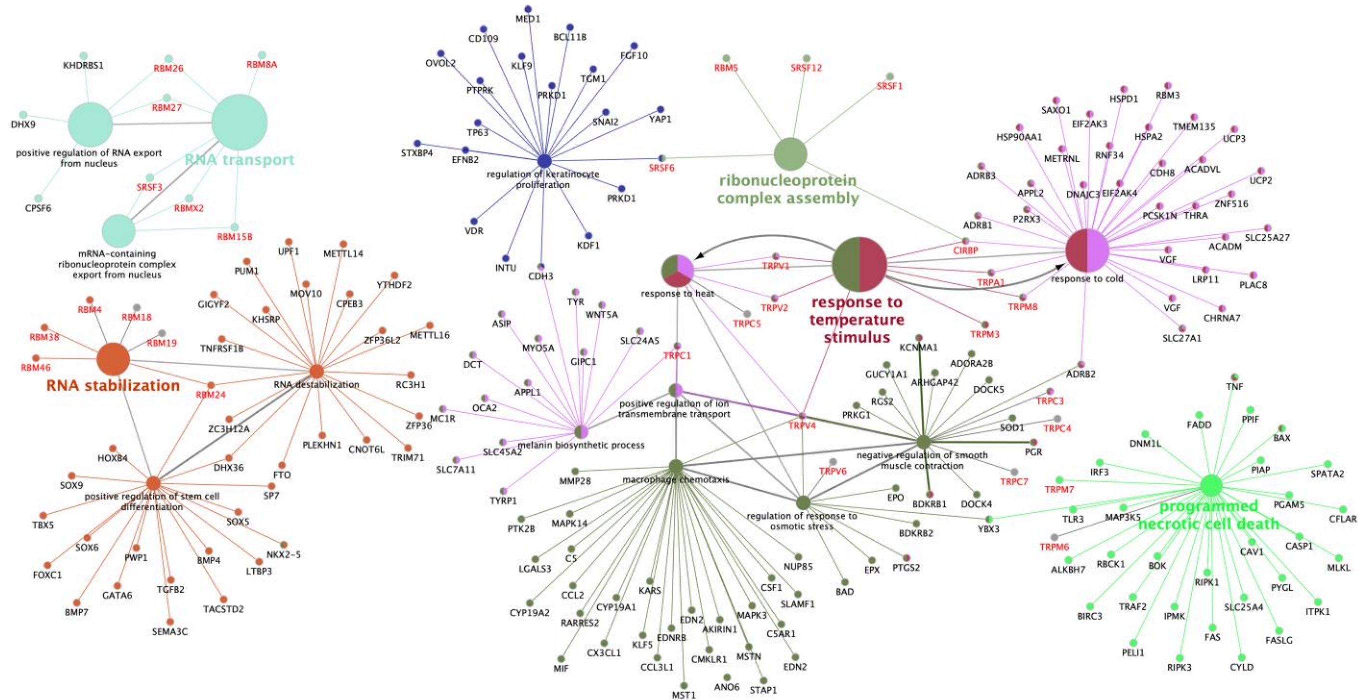


Figure 5. Schematic representation of functionally grouped terms with pathways and genes. Terms and their associated genes share the colour. Genes marked in red are overrepresented in all tissues and treatments. The size of circle corresponds to the p-value for the enrichment. This network was created using the Cytoscape v3.0.0 application and the ClueGO+CluePedia (v. 2.2.5) plug-in. The parameters included: biological process database (BP; date: 28.03.2019); Gene Ontology (GO) tree levels, 1–6 (first level = 0); minimum number of genes, 2; minimum percentage of genes, 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; number of genes included in term <100. The resulting network was modified to delete some redundant and noninformative terms and to manually rearrange the network.

Discussion

Relevant aspects of pig reproduction have recently focused on the differential mRNA expression evolved from the interaction between semen and the female reproductive tract (Alvarez-Rodriguez *et al.* 2019; Álvarez-Rodríguez *et al.* 2020; Martinez *et al.* 2020). However, to the best of our knowledge, the mRNA expression of genes encoding for RBPs and TRP ion channels in the sow reproductive tract is yet to be characterized, particularly whether they are induced by semen or sperm-free SP during the pre-ovulatory phase.

The RBPs are fundamental for RNA metabolism, and thereby essential to gene expression regulation (Bock *et al.* 2015). A group of evolutionarily conserved RBPs, named CIPs, are transcriptionally up-regulated in response to moderately low temperatures as well as other cellular stressors (Zhu *et al.* 2016; Fujita *et al.* 2017). This study explores the differential mRNA expression levels of RBPs and TRP ion channels in the reproductive tract of sows in physiological, spontaneous, stress-free estrus, following cervical deposition of semen or sperm-free SP, without any other manipulation. The study had a special interest in determining whether these cervical depositions affected the differential mRNA expression of CIPs and related TRP ion channels.

A high number of RBPs expressed in the reproductive tract regulate the expression of genes encoding for inflammatory molecules, either promoting or controlling inflammation (Kafasla *et al.* 2014). In the present study, the majority of the DEGs encoding for RBPs were down-regulated in sperm-containing treatments compared to sperm-free SP treatments. This down-regulation could be related to an implication of RBPs on post-transcriptional regulation of immune response against spermatozoa. Thus, this signaling could induce a reduction of the response against spermatozoa retained in specific segments of the female reproductive tract considered reservoirs (Schjenken and Robertson 2020) during the pre-ovulatory phase, keeping spermatozoa safe for further reproductive events. This hypothesis is in agreement with previous research (Alvarez-Rodriguez *et al.* 2019), which reported a down-regulation of genes related to immune pathways in the sow reproductive tract after mating. Additionally, the infundibulum showed greater numbers of up-regulated DEGs encoding for RBPs compared to proximal uterus in all treatments, perhaps related to inflammation-like changes as a pre-amble to an ovulation to be (Duffy *et al.* 2019). A more likely explanation, considering all the sows were in pre-ovulation oestrus stage is the fact that the porcine infundibulum depicts a particular pattern of immune reactivity in relation to sperm transport and elimination through the ostium (Jiwakanon *et al.* 2010).

Information is scarce about the presence of CIRBP in the reproductive tract (Hamid *et al.* 2003) and gonads (Gardela, García-Sanmartín, *et al.* 2019; Gardela, Ruiz-Conca, *et al.* 2019). CIRBP can act as protein component of innate receptors in hypoxic macrophages and brain microglia

interacting with MD2, the coreceptor of Toll-like receptor 4, enhancing the proinflammatory response (Qiang *et al.* 2013; Rajayer *et al.* 2013). Our results reported that mating up-regulated CIRBP mRNA expression in the uterus and the oviductal segments, suggesting that *CIRBP* may be involved in the promotion of the described inflammatory response after mating (Katila 2012), but not in other treatments included in the study. Thus, our data propose that this promotion of inflammation does not result from the separate action of spermatozoa nor SP but perhaps from an intrinsic signaling mechanism maybe produced by the mating process, as it has been postulated in human as a cervico-uterine-tubal reflex (Shafik *et al.* 2006), although never proved in pigs. As per the entry of SP, the bulk of SP does not seem to enter the oviduct of the pig, but SP proteins adsorbed to the spermatozoa do (Calvete *et al.* 1997; Rodríguez-Martínez *et al.* 1998; Caballero *et al.* 2005). CIRBP may be involved in early reproductive processes, but more analyses are needed to clarify its role in the sow reproductive tract during the pre-ovulatory phase.

The SRSFs, which consist of 12 members, have been currently reported to regulate metabolic homeostasis and energy-dependent development (Sen *et al.* 2013; Li *et al.* 2014). The dysregulation of SRSFs has been related to the progression of multiple types of human tumors (Karni *et al.* 2007). Here, we reported differential expression of six genes encoding for SRSFs, including SRSF5, a recently described mammalian CIP (Fujita *et al.* 2017). Our results suggest that SRSFs are involved in the interaction between the female reproductive tract and semen, particularly in the oviduct, where we found a greater number of down-regulated genes encoding for SRSFs in sperm-containing treatments. Curiously, sperm-free treatments up-regulated a greater number of genes encoding for SRSFs in the oviduct, especially in the sperm-free SP of the whole ejaculate group. Further analyses are needed to elucidate the functions of SRSFs genes in the swine female reproductive tract during the pre-ovulatory phase.

In the current study, a common down-regulation of *SRSF1* (also known as SF2/ASF), a SRSFs involved in pre-mRNA splicing (Das and Krainer 2014), was reported between sperm-containing treatments in the ampulla. Even though no information is available about how SRSF1 gene is involved in the context of porcine oocyte quality, less competent oocytes up-regulated SRSF1 (Yuan *et al.* 2011). Taken together, a down-regulation of *SRSF1* might be functionally relevant to oocyte competence in the ampulla, where fertilization takes place (Suarez 2008).

Both SRSF3 (also called SRp20) and SRSF7 (also called 9G8) regulate 3′ untranslated region identity and length in an opposing manner (Müller-McNicoll *et al.* 2016). Our results, mainly the ampullary down-regulation in sperm-containing treatments for *SRSF3* expression, might be related to a decrease in the arrest signaling of oocytes and embryos, as previously studied (Jumaa *et al.* 1999; Do *et al.* 2018). Moreover, SRSF3 and SRSF7 play key roles in both regulating alternative polyadenylation and splicing during mouse oocyte development (Kasowitz *et al.* 2018). Besides, SRSF7 has appeared to inhibit proliferation while enhancing apoptosis (Fu and Wang

2018). Our results regarding *SRSF7* expression were only present when sows were inseminated with the sperm-rich fraction, and solely in the oviductal tissues (utero-tubal junction, isthmus, and ampulla), thus suggesting that mating should repress, through a yet not fully understood mechanism, this apoptotic response inhibition.

The TRP ion channels are expressed in a wide variety of tissues and cells, playing fundamental roles as cellular sensors and signal integrators of an extensive Ca^{2+}-mediated cellular functions maintaining ion homeostasis (Nilius 2007). Investigating the differences in the number of DEGs between the treatments of the study, a greater number of up-regulated DEGs encoding for TRP ion channels were found in NM compared to the sperm-free SP of the whole ejaculate. Considering the multiple stimuli produced by mating and the resulting utero-tubal responses (Shafik *et al.* 2006; Parada-Bustamante *et al.* 2016), it seems logical that a greater number of genes encoding for TRP ion channels were up-regulated compared to its sperm-free SP counterpart. However, at the same time, NM down-regulated a greater number of DEGs encoding for TRP ion channels compared to the rest of the treatments included in this study. This fact suggests that mating may be a complex input that is perceived by the sensory system of the female reproductive tract, including chemo-, thermo-, and mechano-sensing (Damann *et al.* 2008), therefore, producing a wide variety of regulatory mechanisms.

The TRPC family has seven members that can be classified in four subfamilies based on structural and functional homology: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 (González-Cobos and Trebak 2010). All TRPC members forms Ca^{2+}-permeable non-selective cation channels that display both, inward and outward rectification with reversal potentials around 0 mV (González-Cobos and Trebak 2010). The TRPC4/5 channels are activated via phospholipase C-coupled receptors, but the mechanisms of activation are still unclear (Otsuguro *et al.* 2008; Trebak *et al.* 2009). TRPC5 is involved in the control of smooth muscle (González-Cobos and Trebak 2010) and was identified as a cold sensor in neurons (Zimmermann *et al.* 2011). Previous research reported highest *TRPC5* expression in the brain compared to kidney, liver, testis, and uterus (Philipp *et al.* 1998). In the present study, all treatments down-regulated *TRPC5* expression in the proximal uterus. In addition, NM downregulated *TRPC5* expression in all tissue sections. Lower *TRPC5* expression has been reported in myometrium during pregnancy compared to non-pregnant rats (Babich *et al.* 2004). Taking together, our results suggest that the first interaction between seminal fluid and endometrium induces a *TRPC5* down-regulation that may be involved in sperm movement through the entire female reproductive tract and perhaps could persist in myometrium during pregnancy. The involvement of TRPC5 in the control of smooth muscle and regulation of Ca^{2+} entry in myometrial cells of the female reproductive tract remains unclear (Babich *et al.* 2004). However, the role of this myometrium regulation could be related to slow down the progression of the spermatozoa along the female reproductive tract after mating during

the pre-ovulatory phase. In contrast, the utero-tubal junction showed a common up-regulation of *TRPC3* between NM, Semen-AI, and SP-AI.

Like TRPC5, TRPC3 is involved in the control of smooth muscle activity (González-Cobos and Trebak 2010). However, unlike TRPC5, overexpression of TRPC3 was reported in human myometrium during pregnancy and labor, compared to non-pregnant state (Dalrymple *et al.* 2004). In our case, the up-regulation of *TRPC3* could be related to the function of the sperm reservoir performed by the utero-tubal junction (Rodríguez-Martínez *et al.* 2005), where hyperactivated sperm motility enables the displacement of spermatozoa from the sperm reservoir to the site of fertilization during the pre-ovulatory phase.

Even though recent research identified the TRPV3, TRPV4, and TRPM8 as inductors of CIPs (Fujita *et al.* 2017; Fujita *et al.* 2018), no statistical differences were found for *TRPV3* and *TRPV4* expression in our study. Acting as a cold and menthol receptor, TRPM8 was identified in sensory neurons (Peier *et al.* 2002) and highly expressed in several tumors, particularly in prostate neoplastic cells (Voets *et al.* 2007). Nevertheless, the physiological function in non-neuronal tissues is not fully understood (Voets *et al.* 2007). Previous research identified the presence of TRPM8 in spermatozoa, probably involved in sperm acrosome reaction and cell signaling events, such as thermotaxis, chemotaxis, and mechanosensory transduction (De Blas *et al.* 2009; Gibbs *et al.* 2011; Martínez-López *et al.* 2011). Here, we reported a down-regulation of *TRPM8* expression in the isthmus produced by NM. This down-regulation may be related to preserving sperm acrosome reaction in isthmus before fertilization takes place.

The TRPA1 constitutes the only mammalian member of the TRPA ion channel subfamily (Meents *et al.* 2019). The TRPA1 is mainly expressed in sensory neurons as a nociceptive integrator and has been identified in multiple non-neuronal tissues (Fernandes, Fernandes and Keeble, 2012). However, little is known about its expression in the female reproductive tract, only being described in rat and human endometrium to date (Pohóczky *et al.* 2016; Bohonyi *et al.* 2017). Non-neuronal implications of TRPA1 include inflammation, infection, and immunity (Fernandes *et al.* 2012). Although data seems to support that TRPA1 is directly activated by cold (Uchida *et al.* 2017), its involvement in cold sensation remains unclear (Meents *et al.* 2019). Sperm-containing treatments down-regulated its expression in uterus and the lower oviductal segments (only NM), suggesting that spermatozoa may interact with TRPA1 and sensory nerves of the reproductive tract to reduce pain and neurogenic inflammation mechanically produced by mating or insemination catheter.

It seems to be certain that RBPs and TRP ion channels are involved in multiple mechanisms during the pre-ovulation phase of the sow; however, the exact mechanisms produced in the interaction between semen and the female reproductive tract have not yet been elucidated. Thus,

larger, exhaustive studies are needed to clarify the detailed mechanisms of RBPs and TRP ion channels, as well as CIPs and related TRP ion channels, in the animal reproduction field.

Materials and methods

The materials and methods followed in this study were designed according to Alvarez-Rodriguez and collaborators (Alvarez-Rodriguez *et al.* 2019).

Ethics Approval

Animal handling and experimentation were performed following the European Community Directive 2010/63/EU, 22/09/2010, and current Swedish legislation (SJVFS 2017:40). The Regional Committee for Ethical Approval of Animal Experiments (Linköpings Djurförsöksetiska nämnd, Linköping, Sweden) approved the experiments. Permits number 75-12 (10/02/2012), ID1400 (02/02/2018), and Dnr 03416-2020 (26/03/2020).

Animals and Housing Conditions

Five young matured boars (9–11 months) of proven sperm quality (concentration, motility, and morphology) and 20 weaned sows (parity 1-3) of Swedish Landrace breed were obtained from a controlled breeding farm. Animals were kept in individual stalls under controlled light and temperature regimens. Animals had ad libitum access to water and were fed with commercial feedstuff.

Experimental Design

Segments of the female internal genital tract of 20 domestic female pigs (Sus scrofa domestica) were surgically retrieved under general anesthesia 24 h after treatments. mRNA expression analyses were performed on the right side of the tubular reproductive tract (uterine horns and oviducts) segmented into cervix, distal uterus, proximal uterus, utero-tubal junction, isthmus, ampulla, and infundibulum. The females were distributed (n = 4) to one of the following five groups:

a) Control group: females were cervically infused with protein-free BTS extender (50 mL).

b) NM: females were each mated with a single male.

c) Semen-AI: females were artificially inseminated with pooled ejaculate of the first 10 mL portion of the sperm-rich fraction (extended to 50 mL with BTS).

d) SP-TOTAL: females were cervically infused with pools of sperm-free SP of the whole ejaculate (50 mL).

e) SP-AI: females were cervically infused with the sperm-free SP harvested from pooled sperm-peak fractions (50 mL).

Semen and Seminal Plasma Collection

Semen was collected weekly using the gloved-hand method (Susan *et al.* 2010). All ejaculates were evaluated for sperm concentration and motility. Ejaculates with at least 70% of motility and 75% of spermatozoa normal morphology were immediately used for the experiments. The SP was obtained from the whole ejaculate or the sperm-rich fraction after double centrifugation at 1500× *g* for 10 min and checking the absence of sperm cells.

Reproductive Management of Sows

Females were checked for pro-estrus and estrus behavioral signs twice daily using a neighboring boar as teaser and the application of back pressure by experienced staff. Sows presenting standing estrus reflex were assumed to be on the first day of behavioral estrus and were randomly assigned to control or treatment groups. Disposable conventional AI-catheters (Minitüb, Munich, Germany) were used for all cervical inseminations or infusions performed in the study (Atikuzzaman *et al.* 2017).

Tissue Sample Collection

Sows were sedated and induced to general anesthesia (Alvarez-Rodriguez *et al.* 2019) 24 h after mating, insemination, or infusion (pre-/peri-ovulation stage). The blood vessels that irrigate the female reproductive tract were clamped. The reproductive tract was opened to expose the mucosa at specific segments. Mucosal samples were retrieved and immediately plunged into liquid nitrogen (LN_2) and stored in cryotubes at -80 °C until further analyses. The utero-tubal junction was collected as a segment and bisected, one half plunged into LN_2 following careful cleansing with buffered saline solution and the other half fixed in 4% paraformaldehyde solution. The fixed samples were histologically processed to confirm presence of spermatozoa in animals of NM and Semen-AI groups, and alternative sperm absence in animals of SP-TOTAL, SP-AI, and Control groups. The confirmation of presence or absence of spermatozoa was done prior to use the utero-tubal junction tissues for microarrays (Susan *et al.* 2010; Atikuzzaman 2016; Atikuzzaman *et al.* 2017). The number of preovulatory follicles or eventual new ovulation was recorded per sow (22.30 ± 7.29, mean ± SD) without significant differences found between sow-groups.

Microarray Hybridization and Scanning

Total RNA from each genital tract segment was extracted following a TRIzol modified protocol (Atikuzzaman *et al.* 2017). Only samples with RIN values greater than 9 were used for microarray hybridization. cDNA synthesis was performed using GeneChip®Whole Transcript Plus reagent kit (Affymetrix, Santa Clara, CA, USA) and equal amounts of total RNA (250 ng/reaction). To make a cocktail hybridization mix for a single reaction, 3.5 μg of fragmented and labelled single-stranded cDNA (41 μL) were mixed with 109 μL of hybridization master mix. Before loading the array chip (GeneChip® Porcine Gene 1.0 ST Array, Affymetrix Inc., 3420 Central Expressway, Santa Clara, CA 95051, USA) with the hybridization cocktail, an initial incubation of the mix at 99 °C for 5 min was performed following a descent to 45 °C. Then, a total of 130 μL of the cocktail hybridization mix was loaded into each array chip and was incubated at 45 °C under 60 rotations per min, for 16 h. After the incubation, the hybridized cartridge array chip was unloaded and subjected to washing and staining using GeneChip® Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA), to be finally scanned using the Affymetrix GeneChip® Scanner GCS3000 (Affymetrix, Santa Clara, CA, USA).

Microarray Data Analyses and Bioinformatics

Individual microarrays were used for each mucosal area of the female genital tract and animal. The intensity data of each array chip was treated using robust multi-array average normalization, computing average intensity values by background adjustment, quantile normalization among arrays, and finally, log2 transformation for extracting the expression values of each transcript in the probe set, as implemented in the Transcriptome Analysis Console (TAC, version 4.0.) from Affymetrix (Santa Clara, CA, USA). The normalized mRNA expression data was analyzed using a linear model and the empirical Bayes' approach implemented in the package *limma*. The differential expressed transcripts were calculated using a Benjamini–Hochberg false discovery rate ($q < 0.05$) and a principal component analysis-based p-value correction to control for multiple testing to control type-I errors.

The protein analysis through evolutionary relationships (PANTHER) GO classification system (Thomas *et al.* 2003) was used to determine the functions of all DEGs. Pathway analyses were based on the KEGG pathway (Kanehisa and Goto 2000). Biomolecular interaction networks were performed using the Cytoscape software (Shannon *et al.* 2003) (v. 3.0.0.) and the ClueGo Cytoscape plug-in (Bindea *et al.* 2009) (v. 2.0.3.).

The number of DEGs, obtained by direct comparison of all the experimental groups (NM, Semen-AI, SP-TOTAL, SP-AI) with the Control group, were analyzed by two-way ANOVA, followed by Tukey's Multiple Comparison test using the GraphPad software for Windows v.

8.0.2. (GraphPad Software, Inc, La Jolla, CA, USA). A $p < 0.05$ was considered statistically significant.

Conclusions

In conclusion, we identified specific differentially expressed mRNA of genes encoding for CIPs and related TRP ion channels along the sow reproductive tract, suggesting that spermatozoa and seminal plasma differentially modulated both protein families during the pre-ovulatory phase. Despite absence of a clear pattern of mRNA expression regulation for the entire internal sow reproductive tract, the results suggest that these genes may play other functions than those related to cold-shock responses described in the literature, thus involved in animal reproduction probably related to an early signaling mechanism of the female reproductive tract in response to both spermatozoa and SP during the pre-ovulatory phase. Besides, the expression changes observed of other genes encoding for RBPs and TRP ion channels, such as *SRSF1*, *SRSF3*, *SRSF7*, and *TRPC3*, suggest that RBPs and TRP ion channels are involved in multiple mechanisms during the pre-ovulation phase of the sow. These results pave for larger comparative studies to clarify the detailed mechanisms of RBPs and TRP ion channels, as well as CIPs and related TRP ion channels.

Author Contributions: Conceptualization, M.A.-R., H.R.-M. and D.W.; methodology, J.G., C.A.M., M.A.-R. and H.R.-M.; software, J.G., C.A.M. and M.A.-R.; validation, J.G. and M.A.-R.; formal analysis, J.G. and M.A.-R.; investigation, J.G., C.A.M. and M.A.-R.; resources, H.R.-M. and M.A.-R.; data curation, J.G. and M.A.-R.; writing—original draft preparation, J.G.; writing—review and editing, M.R.-C, M.A.-R., H.R.-M., D.W and M.L.-B; visualization, J.G. and C.A.M.; supervision, M.A.-R., H.R.-M. and M.L.-B.; project administration, M.A.-R. and H.R.-M.; funding acquisition, H.R.-M. and M.A.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Research Council FORMAS, Stockholm (Project 2017-00946 and Project 2019-00288), the Swedish Research Council (Vetenskapsrådet, VR; project 2015-05919), the Government of Catalonia-AGAUR co-financed with the European Social Fund (2018 FI_B 00236), the Government of Spain-Ministry of Education, Culture and Sports (Training programme for Academic Staff FPU15/06029), and the Seneca Foundation (20780/PD/18), Murcia (Spain).

Acknowledgments: Annette Molbaek and Åsa Schippert, from genomics Core Facility at Linköping University for expert assistance when running the microarrays. To Linköping University for supporting the open access funding.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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GENERAL DISCUSSION

Oocytes remain at a very early stage of maturation when females are born (Adams and Singh 2015). When reaching puberty, a set of oocytes is selected at each estrous cycle to progress (Estill 2015). Females do not generate new gametes during their reproductive years as males do, so it was thought until recently (Tilly *et al.* 2009).

In cattle, one oocyte is usually ovulated at each ovulation period, whereas the rest of the cohort degenerates (Adams and Singh 2015). Oocytes need to reach the metaphase II stage to be fertilized; otherwise, the chances of fertilization are meager (Duranthon and Renard 2001; Wrenzycki and Stinshoff 2013). *In vitro* maturation procedures are needed when handling immature oocytes and are crucial for cryopreservation (Borini and Bianchi 2010). Cryopreservation of immature states is less likely to obtain good performance in maturation, fertilization, and subsequent embryo development in cattle (Diez *et al.* 2005). As a result, combining oocyte cryopreservation with *in vitro* maturation is critical for improving the survival rate of vitrified oocytes and their developmental competence.

However, reproductive success is not just about the quality of the oocytes after vitrification and fertilization; it is also about the interplay between several physiological factors (i.e., hormonal control, cytokines, and immune cells, among others) (Wira *et al.* 2005). The changes that take place in the female reproductive tract during all the reproductive events are crucial to success (Estill 2015). Further comprehension of the complexities of the female physiological adaptations and examining the interaction between the female reproductive system and other factors in reproductive physiology is fundamental.

Differential CIRBP expression in oocytes and cumulus cells induced by mild hypothermia

Mild hypothermia induces the expression of a group of RNA-binding proteins known as cold-inducible RNA-binding proteins (Derry *et al.* 1995; Danno *et al.* 1997; Nishiyama *et al.* 1997; Tong *et al.* 2013; Rzechorzek *et al.* 2015; Fujita *et al.* 2017). CIRBP is one of the most studied members of the cold-inducible protein family (Zhong and Huang 2017). Due to its roles in cell survival and anti-apoptotic cascades (Zhu *et al.* 2016), CIRBP is a potential candidate to improve bovine oocyte cryotolerance to vitrification.

Accordingly, our first aim was to evaluate whether the exposure of sublethal mild hypothermia during *in vitro* maturation of bovine cumulus-oocyte complexes represents a potential strategy to

increase the expression of CIRBP in oocytes and cumulus cells. Previously reported in yak (*Bos grunniens*), the increase of CIRBP has been linked with enhancing the developmental competence of vitrified/warmed oocytes (Pan *et al.* 2015). However, no direct mild hypothermia was applied during *in vitro* maturation of those oocytes. When applied directly to *Xenopus laevis* oocytes, no changes in xCIRP2 (mammalian CIRBP homolog protein) expression were reported (Matsumoto *et al.* 2000).

Effects of mild hypothermia during *in vitro* maturation

Our first approach was based on applying sublethal mild hypothermia during *in vitro* maturation of bovine oocytes (Chapter I). One of our primary concerns was the presence of detrimental effects on the nuclear maturation and cytoplasmic cortical granule distribution of oocytes due to the sublethal stress. The results confirmed that sublethal mild hypothermia negatively affected the optimal competence of the *in vitro* matured bovine oocytes.

However, we reported an increase in the protein expression of CIRBP in the cold stressed cumulus-oocyte complexes, exclusively expressed by the cumulus cells. Because of the bidirectional communication between the cumulus cells and the oocytes (Dumesic *et al.* 2015; Russell *et al.* 2016), the increase of CIRBP in cumulus cells may play an essential role during the maturation of the oocyte and the vitrification procedure.

Any alternative to evade the cellular damage induced by mild hypothermia?

Due to the detrimental effects reported in our first approach, we then explored other methods to avoid the detrimental effects of sublethal mild hypothermia during *in vitro* maturation of bovine oocytes while increasing the expression of CIRBP.

A second study was performed using exogenous CIRBP and the small molecule zr17-2 to mimic a cold stress response in the absence of a cold stimulus (Chapter II). Therefore, our second aim was to assess the supplementation of exogenous CIRBP and zr17-2 during *in vitro* maturation of bovine cumulus-oocyte complexes as a strategy to increase *CIRBP* mRNA expression in oocytes and cumulus cells prior to vitrification. The similar transcriptome changes observed in oocytes and cumulus cells when exposed to mild hypothermia may suggest a strong communication between both cell types and shared stress response. Additionally, our results identified statistical differences in the *CIRBP* mRNA expression when cumulus-oocyte complexes were exposed to mild hypothermia, but not when exposed to the exogenous CIRBP and zr17-2, or even the vitrification itself. The expression of CIRBP has been previously used as a cryoinjury marker after slow-freezing or vitrification of oocytes and embryos (Shin *et al.* 2011; Wen *et al.* 2014; Jo *et al.* 2015), but our data disagree with their results.

Understanding how CIRBP and other CIPs affect oocyte maturation could lead to developing of methodologies that can protect oocytes against vitrification-induced damage. In this regard, more research is needed to evaluate if the reported mRNA expression variations impact on overall oocyte quality and, maybe, embryo development.

Differential CIRBP expression in the female reproductive tract

The cyclic changes of the female reproductive tract are crucial to provide an optimal environment for each reproductive event (Brenner and West 1975). Several endometrial transcriptome alterations have been discovered in cattle at various periods of the estrous cycle (Forde *et al.* 2011). In response to a wide range of stimuli, several gene regulatory processes are engaged to coordinate an optimal cellular response (Harvey *et al.* 2017). The post-transcriptional control of RNAs is an essential regulator of gene expression (Änkö 2018), mainly achieved by the RNA-binding proteins (Lunde *et al.* 2007). The post-transcriptional regulation performed by the RNA-binding proteins is essential in tissue homeostasis and pathophysiology in multiple tissues, including the female reproductive tract (Khalaj *et al.* 2017; Änkö 2018). However, the exact process by which the RNA-binding proteins are controlled in the reproductive tissues is still unknown. As an RNA-binding protein, CIRBP is a potential candidate to study its modulation in the physiology of the female reproductive tract.

Modulation by the estrous cycle

In the present thesis, we aimed to determine the endometrial and ampullary changes in CIRBP expression during the stages of the bovine estrous cycle. Our results demonstrate that the CIRBP expression changes in both tissues during the bovine estrous cycle (Chapter III). Specifically, an increase in protein and mRNA expression of CIRBP was observed during stage III of the bovine estrous cycle (Days 11-17) in the endometrium and ampulla. A previous study demonstrated elevated protein level expression of CIRBP during the secretory (luteal) phase in the human uterus (Hamid *et al.* 2003). Moreover, the latter study revealed that the levels of CIRBP were negatively associated with endometrial gland proliferative activity (Hamid *et al.* 2003). The exact functions of CIRBP during the estrous cycle are unknown but probably may be related to the regulation of the endometrial cell cycle (Hamid *et al.* 2003).

In the endometrium, two cell types are present: glandular and stromal cells (Ohtani *et al.* 1993). At each estrous cycle, the bovine endometrium is remodeled, inducing histology changes (Ohtani *et al.* 1993) and thickness modifications (Pierson and Ginther 1987; Sugiura *et al.* 2018). The endometrial thickness increases at 3-4 days before ovulation (Days 17-18) and decreases 1 day

before (Day 20) to 3 days after ovulation (Day 3), then reaching the basal level of the luteal phase (Pierson and Ginther 1987; Sugiura *et al.* 2018). The histological changes regarding cell proliferation in the bovine endometrium are characterized by subtle changes after the mid-luteal phase (Ohtani *et al.* 1993). Stromal mitoses, which are prominent between Days 9 and 10, abruptly decrease by Day 13 until Day 15, and on Day 18, few glandular mitoses reappear, which remain until Day 5 (Ohtani *et al.* 1993).

To date, no previous studies have specifically evaluated the CIRBP expression in the ampulla of any species. This thesis is, therefore, the first to reveal that CIRBP expression is modulated in the bovine ampulla during the estrous cycle (Chapter III). However, as for endometrium, the biological impact of these changes is still to unveil. Notwithstanding, we suggest that CIRBP may be related to the cell cycle regulation of the ampullary epithelium, probably following the ampullary histological changes during the estrous cycle. Two types of cells are present in the oviduct: ciliated cells, which are essential for oocyte and embryo transportation, and secretory cells, which produce factors to promote further embryonic development and survival (Killian 2004). The bovine ampullar epithelium is higher during the follicular phase than the luteal phase, with a predominance of ciliated cells, whereas secretory cells and their activity are predominant during the luteal phase (Mokhtar 2015). This epithelial remodeling and change in proportions of ciliated and secretory cells during the bovine estrous cycle is characterized by secretory cell differentiation and proliferation (Ito *et al.* 2016). Mitosis of secretory cells increases during the follicular phase (Days 19-21) and the day of ovulation (Day 0) and decreases at mid and late luteal phases (Days 8-12 and Days 15-17) (Ito *et al.* 2016). As previously hypothesized in the human endometrium, in which CIRBP was negatively associated with endometrial gland proliferative activity (Hamid *et al.* 2003), we suggest that CIRBP may regulte the bovine endometrial and ampullar epithelium changes.

Together, these results provide new molecular insights of CIRBP during the bovine estrous cycle and raise questions about the CIRBP regulation and functions in non-hypothermic conditions. Nevertheless, more research is needed before being used as potential biomarkers for reproductive physiology performance and to clarify the role of CIRBP in the endometrial and ampullar epithelium.

Modulation by the male-female interaction

Another necessary modulation process takes place when the ejaculate is deposited into the female reproductive tract, causing mRNA and protein expression changes in the endometrial and oviductal tissues of different species (Orihuela *et al.* 2001; Bauersachs *et al.* 2003; Fazeli *et al.* 2004; Georgiou *et al.* 2007; Almiñana *et al.* 2014; Álvarez-Rodríguez *et al.* 2020; Gardela *et al.*

2020a; Gardela *et al.* 2020b), modulating angiogenesis, sperm storage and selection, oviduct contractility, oocyte transfer, and preimplantational embryo development.

Besides confirming the modulation of CIRBP during the bovine estrous cycle, we aimed to examine whether the male-female interaction modulates the CIRBP mRNA expression in the porcine model. Our results demonstrated changes in the mRNA expression of CIRBP and other RNA-binding proteins in the sow reproductive tract in response to mating and seminal infusions (Chapter IV). This thesis is the first report that reveals the potential roles of CIRBP and other RNA-binding proteins in animal reproductive physiology to the best of our knowledge. As previously discussed, the exact mechanisms and roles of CIRBP in the female reproductive tract remain to be elucidated. The presence of CIRBP in the reproductive tract is poorly understood (Hamid *et al.* 2003; Gardela, García-Sanmartín, *et al.* 2019; Gardela, Ruiz-Conca, *et al.* 2019); however, we can hypothesize potential roles in reproductive physiology according to previous results in other research fields.

After mating, an early inflammatory response is induced by sperm and seminal components (Katila 2012). The immune response originated creates a suitable environment for embryo survival, implantation success, optimal fetal and placental development, and the overall reproductive process (Hansen 2011). Linked with immune responses, CIRBP plays a crucial role in mediating inflammatory processes (Zhu *et al.* 2016; Liao *et al.* 2017; Zhong and Huang 2017). Several studies showed that extracellular CIRBP is an endogenous pro-inflammatory mediator and damage-associated molecular pattern that activates inflammatory responses in hemorrhagic shock and sepsis (Brochu *et al.* 2013; Qiang *et al.* 2013; Aziz *et al.* 2019). Additionally, CIRBP regulates inflammation through macrophages and the Toll-like receptor 4 (Khan *et al.* 2017; Li *et al.* 2017; Bolourani *et al.* 2021) and can induce vascular damage by activating the NLRP3 inflammasome pathway (Yang *et al.* 2016). In acute systemic inflammatory conditions, CIRBP is involved in several inflammation-related diseases (Rajayer *et al.* 2013; Zhou *et al.* 2014; Godwin *et al.* 2015; Cen *et al.* 2016; Li *et al.* 2016; Hozumi *et al.* 2021), suggesting a complex role in mediating inflammatory process, which may be cell or tissue-specific.

According to our results, mating increased CIRBP mRNA expression in the uterine and oviductal segments but not in the other treatments tested, semen or seminal plasma infusions (Chapter IV). These results suggest that CIRBP may play a role in enhancing the inflammatory response observed after mating (Katila 2012). Additionally, our data suggest that this supposed role of CIRBP in the post-mating inflammatory response is due to an innate signaling mechanism induced by the mating process itself rather than an action of sperm or seminal plasma (Chapter IV). Thus, we encourage further research in this area to better understand whether and how CIRBP is involved in the post-mating inflammatory response, the pre-ovulatory phase, and other reproductive events.

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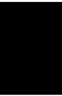
CONCLUSIONS



The studies performed in the present thesis were conducted with the aim of providing insights into the role of CIRBP in the regulation of domestic animal reproduction and how its modulation can benefit assisted reproduction techniques.

The following conclusions can be drawn accordingly:

Specific objective 1

To evaluate whether the exposure of bovine cumulus-oocyte complexes to sublethal mild hypothermia during *in vitro* maturation may represent a potential strategy to increase CIRBP expression in oocytes and cumulus cells.

Conclusion 1.1

Protein expression of CIRBP is increased in response to sublethal mild hypothermia during *in vitro* maturation of bovine cumulus-oocyte complexes.

Conclusion 1.2

Sublethal mild hypothermia negatively affects the competence of bovine oocytes during *in vitro* maturation, shown by the optimal percentage of both nuclear maturation and cytoplasmic cortical granule distribution.

Specific objective 2

To assess the supplementation of exogenous CIRBP and the small molecule zr17-2 during *in vitro* maturation of bovine cumulus-oocyte complexes as an approach to increase CIRBP expression in oocytes and cumulus cells prior to vitrification.

Conclusion 2.1

The exogenous supplementation with zr17-2 to the maturation media has little effect on *CIRBP* mRNA expression of both oocytes and cumulus cells, affecting *IL-1β* mRNA expression under physiological temperatures and at the higher concentration assayed.

Conclusion 2.2

Sublethal mild hypothermia during *in vitro* maturation increases *CIRBP* mRNA expression in cumulus and oocytes.

Specific objective 3

To determine the endometrial and ampullary changes in CIRBP expression during the stages of the bovine estrous cycle.

Conclusion 3.1

Protein and mRNA expression of CIRBP in the endometrium and ampulla change during the bovine estrous and correlate accordingly, displaying higher levels in both tissues during the late luteal phase of the bovine estrous cycle (Days 11-17).

Conclusion 3.2

Changes in the mRNA expression of other cold-inducible proteins (*RBM3* and *SRSF5*), a pro-inflammatory cytokine (*IL-1β*), and other related genes involved in inflammatory pathways (*IL-10RA*, *IL-10RB*, *BCL2*, *NLRP3*, *STAT1*, *STAT3*, *STAT5A*, *STAT6*) are detected across the bovine estrus cycle.

Specific objective 4

To examine whether the male-female interaction modulates CIRBP expression in the reproductive tract of peri-ovulatory sows.

Conclusion 4.1

CIRBP mRNA expression of the sow reproductive tract changes in response to the male-female interaction, revealing that this modulation could be potentially linked to the post-mating inflammatory response.

Conclusion 4.2

Mating increases the *CIRBP* mRNA expression in the uterine and oviductal segments of the sow reproductive tract but not the infusion of semen or seminal plasma.

Conclusion 4.3

The findings on the differential mRNA expression on other RNA-binding proteins and transient potential ion channels along the sow female reproductive tract, especially to cold-inducible proteins and related transient potential ion channels, state that spermatozoa and seminal plasma differentially modulate the transcript expression during the preovulatory phase through a still unknown early signaling mechanism.