Research in Veterinary Science 152 (2022) 181–189

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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

Jaume Gardela [a,b,*], Mateo Ruiz-Conca [a,b], Sergi Olvera-Maneu [b], Manel López-Béjar [b,c], Manuel Álvarez-Rodríguez [a,b]

[a] Division of Children's and Women Health (BKH), Obstetrics and Gynecology, Department of Biomedical and Clinical Sciences (BKV), Linköping University, 58185, Linköping, Sweden
[b] Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, 08193, Bellaterra, Spain
[c] College of Veterinary Medicine, Western University of Health Sciences, Pomona, CA 91766, USA

ARTICLE INFO

Keywords:
Cold-inducible RNA-binding protein
qPCR
Western blot
Cattle

ABSTRACT

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 CIPs in reproductive events raise questions about their role 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 the fertility rate.

1. 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

* Corresponding author at: Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, 08193, Bellaterra, Spain.
E-mail address: jaume.gardela@uab.cat (J. Gardela).

https://doi.org/10.1016/j.rvsc.2022.08.006
Received 3 December 2021; Received in revised form 8 July 2022; Accepted 6 August 2022
Available online 12 August 2022

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 et al., 2019b), and it is localized in the bovine ovary (Gardela et al., 2019a) 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 (Bin 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) (Bin and Sharma, 2015). Binding IL-10 to its receptor inhibits autophagy through the phosphoinositide-3 K (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*, *STAT3, STAT5A, STAT6*).

2. Material and methods

2.1. Animals and tissue collection

Commercial crossbreed beef postpubertal heifers (*Bos taurus*) aged between 13 and 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, <1 h from the harvest of the female reproductive tracts. Additionally, a section of the ampulla of the ipsilateral oviduct was obtained for each animal. Only the epithelial folds on the inner surface of the ampulla were processed for RNA extraction. 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.

2.2. 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 5000 X 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.

2.3. 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 et al., 2020a). The homogenized was centrifuged (12,000 X g, 10 min, 4 °C) and the supernatant was retrieved and mixed with bromochloropropane (100 μL/mL homogenized). After centrifugation (12,000 X 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 X g, 10 min, 4 °C) and the supernatants were discarded. For each sample, 1 mL 75% ethanol was added. Supernatants were discarded after centrifugation (7500 X 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.

2.4. Quantitative real-time PCR analyses

Following a previous protocol for quantitative real-time polymerase chain reaction (qPCR) (Gardela et al., 2020a) (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 Supplementary Table S1 and were confirmed by loading the amplicons in an agarose gel using a gel imaging system (ChemiDoc XRS+ System, Bio-Rad Laboratories, Inc., Kabelsketal, Germany).

2.5. 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 X 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 1:1000 in the same membranes. To visualize immunoreactivity, membranes were incubated with secondary antibody donkey anti-rabbit IgGs (IRDye® 800 CW-labelled, 926–32,213, 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).

2.6. 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. Additionally, to investigate whether the CIRBP protein abundance occurs in proportion to its mRNA relative levels, Spearman's rank correlation coefficients were performed.

3. Results

3.1. 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 (Fig. 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

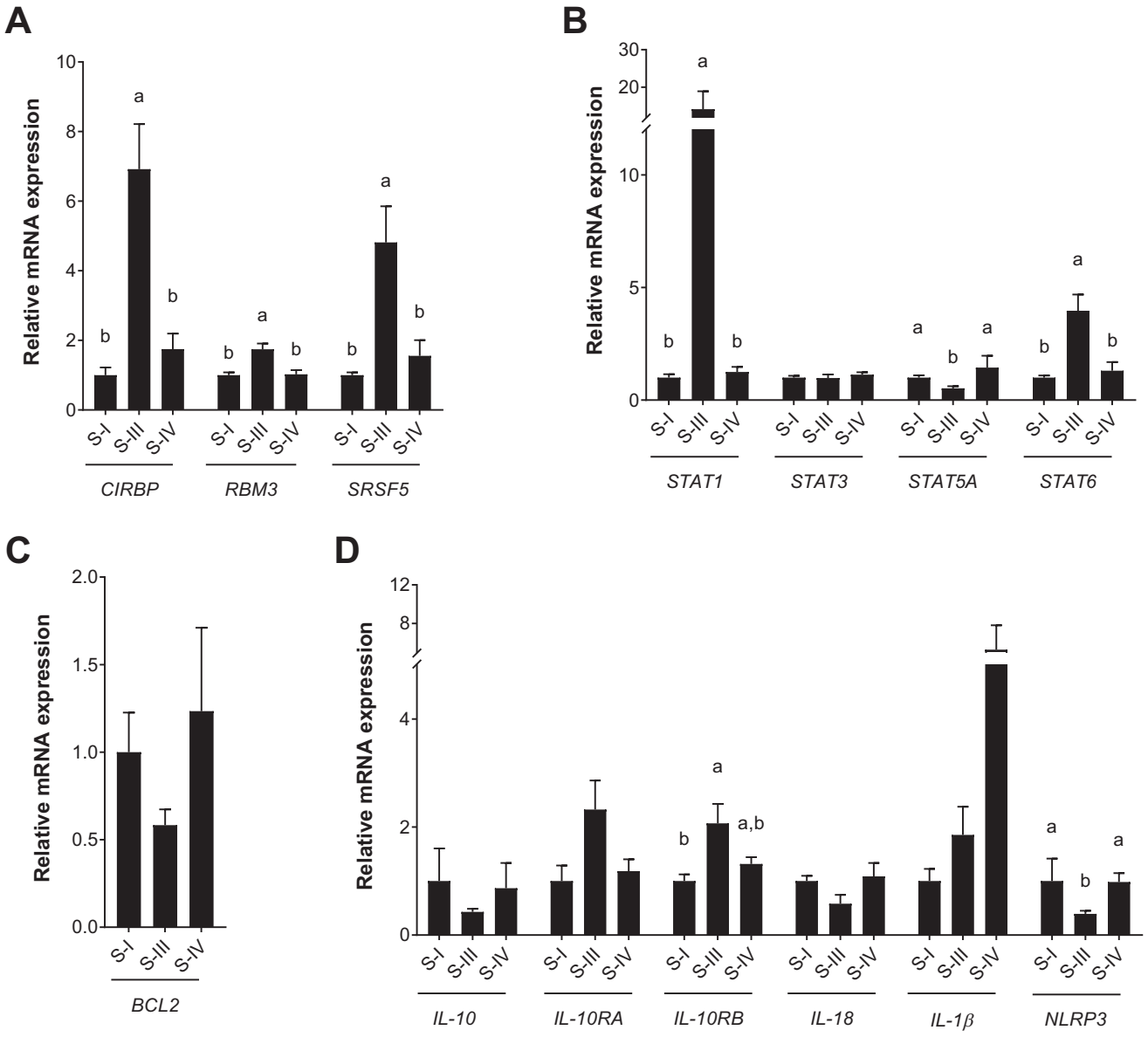

Fig. 1. Endometrial relative mRNA expressions changes of the genes included in the study. Genes involved in (A) cold-inducible proteins, (B) factors of the signal transducer and activator of transcription (STAT) family, (C) the anti-apoptotic gene *BCL2*, and (D) inflammation, comparing stage I (S-I, $n = 6$), 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.

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 ($P < 0.05$).

3.2. 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 (Fig. 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 ($P < 0.05$).

3.3. 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 (Fig. 3A). The progesterone/estradiol (P4/E2) ratio did not differ significantly between the stages of the estrous cycle (Fig. 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 (Supplementary Fig. 1) and the ampulla (Supplementary Fig. 2). 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

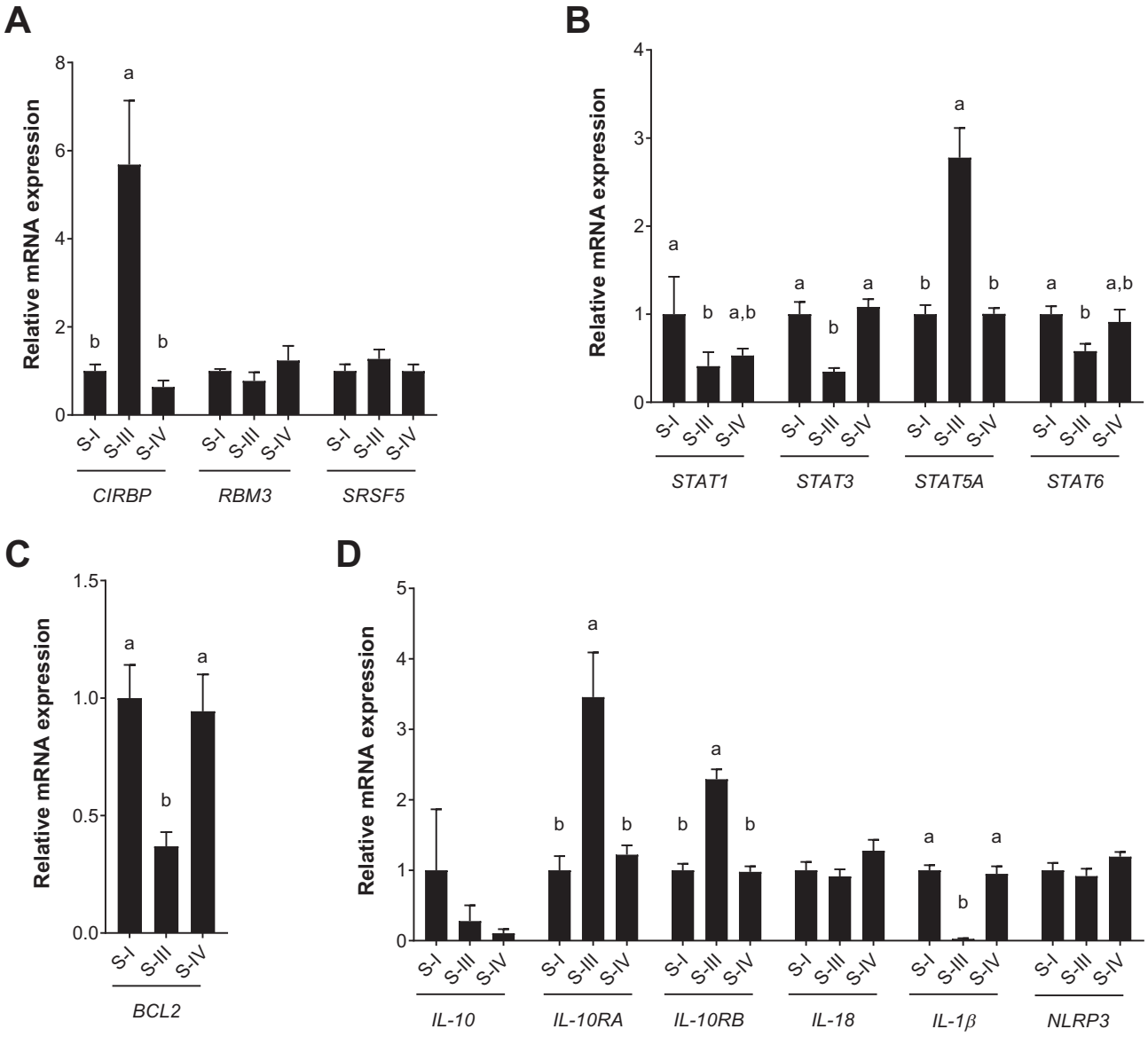

Fig. 2. Ampullary relative mRNA expressions changes of the genes included in the study. Genes involved in (A) cold-inducible proteins, (B) factors of the signal transducer and activator of transcription (STAT) family, (C) the anti-apoptotic gene *BCL2*, and (D) inflammation, comparing stage I (S-I, n = 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.

interleukins and their receptors, and with *NLRP3* during stage III.

3.4. 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 (Fig. 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 stage III compared to stage IV ($P < 0.05$).

3.5. 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 (Fig. 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.

4. 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 et al., 2020b). 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

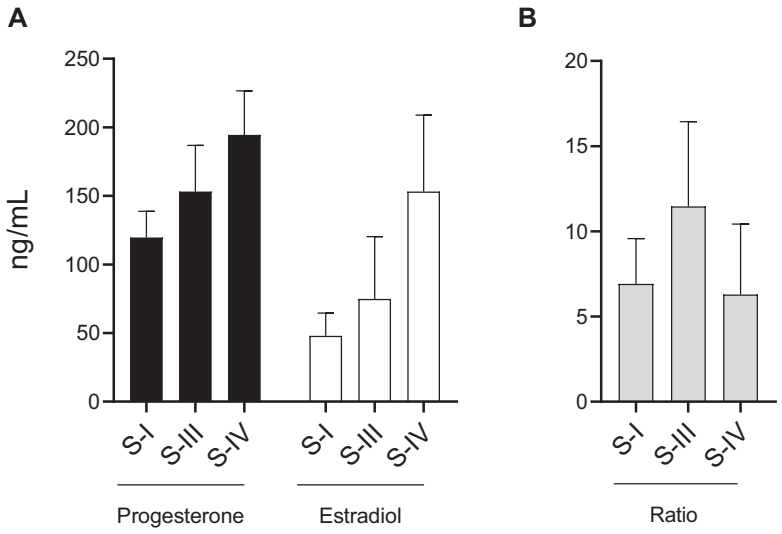

Fig. 3. Follicular fluid hormone concentrations and ratio progesterone/estradiol. (A) Follicular fluid progesterone and estradiol concentrations (ng/mL) and (B) ratio progesterone/estradiol during stage I (S-I, n = 6), stage III (S-III, n = 8), and stage IV (S-IV, n = 6) of the bovine estrus cycle. Non-significant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

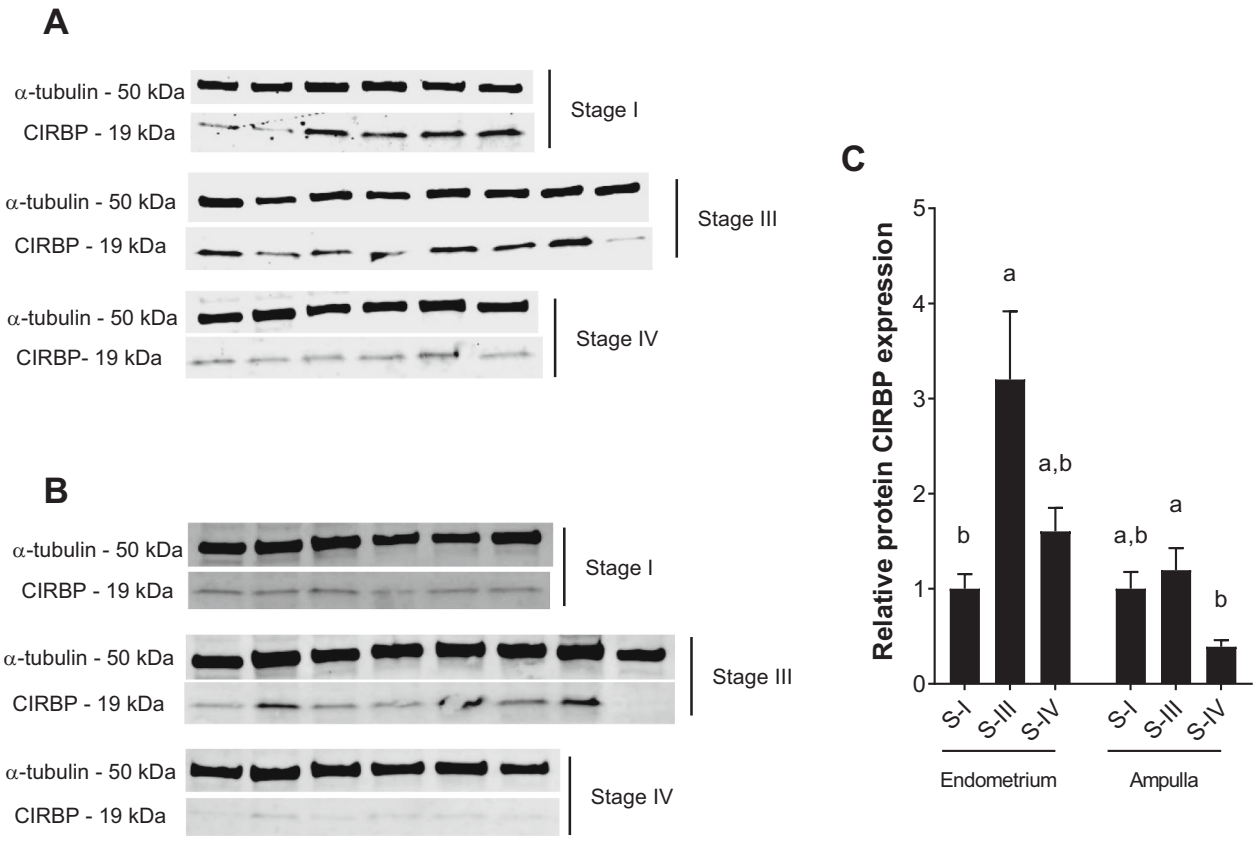

Fig. 4. Relative CIRBP protein expression in endometrium and ampulla. Western blot analyses of relative CIRBP protein expression in (A) the endometrium and (B) ampulla. (C) Relative protein expression of CIRBP in the endometrium and the ampulla, comparing stage I (S-I, n = 6), stage III (S-III, n = 8), 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.

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

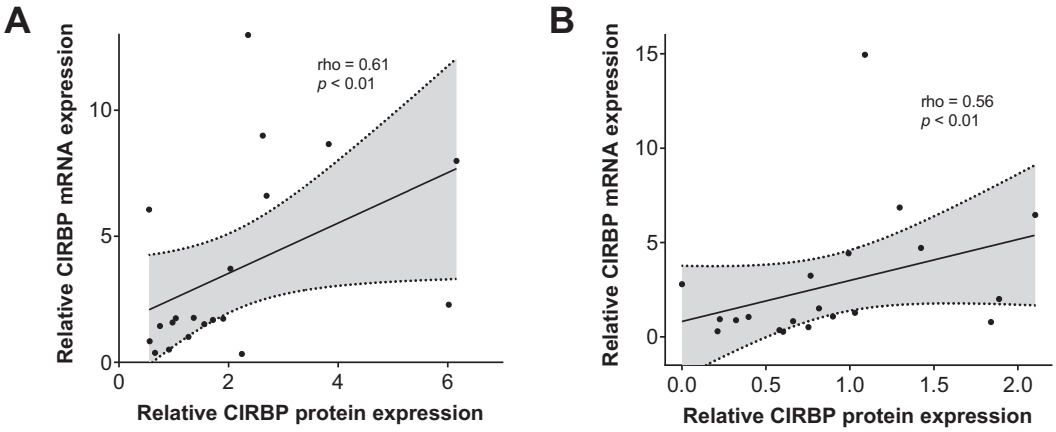

Fig. 5. Correlation between relative CIRBP mRNA and protein expression. Spearman rank correlation scatter plots and linear regression with its 95% confidence interval (gray area) of the relative *CIRBP* mRNA expression and the CIRBP protein expression (A) in the endometrium and (B) ampulla.

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, the exact mechanism behind these results 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 (Fischer et al., 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.

5. 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.08.006.

Ethics approval

Not applicable.

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).

CRediT authorship contribution statement

Jaume Gardela: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Mateo Ruiz-Conca:** Methodology, Investigation, Writing – review & editing. **Sergi Olvera-Maneu:** Methodology, Validation, Investigation, Writing – review & editing. **Manel López-Béjar:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Manuel Álvarez-Rodríguez:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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.

References

Änkö, M.-L., 2018. RNA processing in health and disease. Semin. Cell Dev. Biol. 79, 78–79.
Aziz, M., Brenner, M., Wang, P., 2019. Extracellular CIRP (eCIRP) and inflammation. J. Leukoc. Biol. 106 (1), 133–146.
Bauersachs, S., Ulbrich, S.E., Gross, K., Schmidt, S.E.M., Meyer, H.H.D., Einspanier, R., Wenigerkind, H., Vermehren, M., Blum, H., Sinowatz, F., et al., 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J. Mol. Endocrinol. 34 (3), 889–908.
Bin, Cheng S., Sharma, S., 2015. Interleukin-10: a pleiotropic regulator in pregnancy. Am. J. Reprod. Immunol. 73 (6), 487–500.
Bowman, T., Garcia, R., Turkson, J., Jove, R., 2000. STATs in oncogenesis. Oncogene 19 (21), 2474–2488.
Bromberg, J.F., 2001. Activation of STAT proteins and growth control. BioEssays 23 (2), 161–169.
Broz, P., Dixit, V.M., 2016. Inflammasomes: mechanism of assembly, regulation and signalling. Nat. Rev. Immunol. 16 (7), 407–420.
Carvalho, A.V., Eozenou, C., Healey, G.D., Forde, N., Reinaud, P., Chebrout, M., Gall, L., Rodde, N., Padilla, A.L., Delville, C.G., et al., 2016. Analysis of STAT1 expression and biological activity reveals interferon-tau-dependent STAT1-regulated SOCS genes in the bovine endometrium. Reprod. Fertil. Dev. 28 (4), 459–474.
Chang, H.Y., Sneddon, J.B., Alizadeh, A.A., Sood, R., West, R.B., Montgomery, K., Chi, J. T., Van De Rijn, M., Botstein, D., Brown, P.O., 2004. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol. 2 (2), 206–214.
Cicchese, J.M., Evans, S., Hult, C., Joslyn, L.R., Wessler, T., Millar, J.A., Marino, S., Cilfone, N.A., Mattila, J.T., Linderman, J.J., et al., 2018. Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. Immunol. Rev. 285 (1), 147–167.
Ciuzan, O., Hancock, J., Pamfil, D., Wilson, I., Ladomery, M., 2015. The evolutionarily conserved multifunctional glycine-rich RNA-binding proteins play key roles in development and stress adaptation. Physiol. Plant. 153 (1), 1–11.
Danno, S., Nishiyama, H., Higashitsuji, H., Yokoi, H., Xue, J.-H., Itoh, K., Matsuda, T., Fujita, J., 1997. Increased transcript level of RBM3, a member of the Glycine-rich RNA-binding protein family, in human cells in response to cold stress. Biochem. Biophys. Res. Commun. 236 (3), 804–807.
Dinarello, C., 1996. Biologic basis for interleukin-1 in disease. Blood 87 (6), 2095–2147.
Donnelly, R.P., Dickensheets, H., Finbloom, D.S., 1999. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. J. Interf. Cytokine Res. 19 (6), 563–573.
Ellington, J.E., 1991. The bovine oviduct and its role in reproduction: a review of the literature. Cornell Vet. 81 (3), 313–328.
Estill, C.T., 2015. Initiation of puberty in heifers. In: Hopper, R.M. (Ed.), Bovine Reproduction. First Edit. Wiley Balckwell, Oxford, UK, pp. 195–202.
Fielding, C.A., McLoughlin, R.M., McLeod, L., Colmont, C.S., Najdovska, M., Grail, D., Ernst, M., Jones, S.A., Topley, N., Jenkins, B.J., 2008. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. J. Immunol. 181 (3), 2189–2195.
Fischer, C., Drillich, M., Odau, S., Heuwieser, W., Einspanier, R., Gabler, C., 2010. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. Reprod. Fertil. Dev. 22 (5), 818–829.
Forde, N., Beltman, M.E., Duffy, G.B., Duffy, P., Mehta, J.P., Ó'Gaora, P., Roche, J.F., Lonergan, P., Crowe, M.A., 2011. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. Biol. Reprod. 84 (2), 266–278.
Fortes, M.R.S., Reverter, A., Nagaraj, S.H., Zhang, Y., Jonsson, N.N., Barris, W., Lehnert, S., Boe-Hansen, G.B., Hawken, R.J., 2011. A single nucleotide polymorphism-derived regulatory gene network underlying puberty in 2 tropical breeds of beef cattle. J. Anim. Sci. 89 (6), 1669–1683.
Fujita, T., Hiroaki, Higashitsuji, Hisako, Higashitsuji, Liu, Y., Itoh, K., Sakurai, T., Kojima, T., Kandori, S., Nishiyama, H., Motoi, Fukumoto, et al., 2017. TRPV4-dependent induction of a novel mammalian cold-inducible protein SRSF5 as well as CIRP and RBM3. Sci. Rep. 7 (1), 2295.
Gabler, C., Fischer, C., Drillich, M., Einspanier, R., Heuwieser, W., 2010. Time-dependent mRNA expression of selected pro-inflammatory factors in the endometrium of primiparous cows postpartum. Reprod. Biol. Endocrinol. 8 (1), 152.
Gardela, J., García-Sanmartín, J., Ruiz-Conca, M., Álvarez-Rodríguez, M., Martínez, A., Mogas, T., López-Béjar, M., 2019a. Immunohistochemical identification of CIRBP in bovine ovary and testicle. Anim. Reprod. 16 (3), 727.
Gardela, J., Ruiz-Conca, M., Álvarez-Rodríguez, M., Mogas, T., López-Béjar, M., 2019b. Induction of CIRBP expression by cold shock on bovine cumulus–oocyte complexes. Reprod. Domest. Anim. 54 (S4), 82–85.
Gardela, J., Jauregi-Miguel, A., Martinez, C.A., Rodriguez-Martinez, H., Lopez-Bejar, M., Alvarez-Rodriguez, M., 2020a. Semen modulates the expression of NGF, ABHD2, VCAN, and CTEN in the reproductive tract of female rabbits. Genes (Basel) 11 (7), 758.
Gardela, J., Ruiz-Conca, M., Martinez, C.A., Wright, D., López-Béjar, M., Rodriguez-Martinez, H., Alvarez-Rodriguez, M., 2020b. The expression of cold-inducible RNA-binding protein mRNA in sow genital tract is modulated by natural mating, but not by seminal plasma. Int. J. Mol. Sci. 21 (15), 5333.
Gärtner, M.A., Peter, S., Jung, M., Drillich, M., Einspanier, R., Gabler, C., 2016. Increased mRNA expression of selected pro-inflammatory factors in inflamed bovine endometrium in vivo as well as in endometrial epithelial cells exposed to Bacillus pumilus in vitro. Reprod. Fertil. Dev. 28 (7), 982–994.
Hamid, A.A., Mandai, M., Fujita, J., Nanbu, K., Kariya, M., Kusakari, T., Fukuhara, K., Fujii, S., 2003. Expression of cold-inducible RNA-binding protein in the Normal endometrium, endometrial hyperplasia, and endometrial carcinoma. Int. J. Gynecol. Pathol. 22 (3), 240–247.

Harvey, R., Dezi, V., Pizzinga, M., Willis, A.E., 2017. Post-transcriptional control of gene expression following stress: the role of RNA-binding proteins. Biochem. Soc. Trans. 0, BST20160364.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50 (3), 346–363.
Ireland, J.J., Murphee, R.L., Coulson, P.B., 1980. Accuracy of predicting stages of bovine estrous cycle by Gross appearance of the Corpus luteum. J. Dairy Sci. 63 (1), 155–160.
Jabbour, H.N., Sales, K.J., Catalano, R.D., Norman, J.E., 2009. Inflammatory pathways in female reproductive health and disease. Reproduction 138 (6), 903–919.
Kafasla, P., Skliris, A., Kontoyiannis, D.L., 2014. Post-transcriptional coordination of immunological responses by RNA-binding proteins. Nat. Immunol. 15 (6), 492–502.
Khalaj, K., Miller, J.E., Fenn, C.R., Ahn, S., Luna, R.L., Symons, L., Monsanto, S.P., Koti, M., Tayade, C., 2017. RNA-binding proteins in female reproductive pathologies. Am. J. Pathol. 187 (6), 1200–1210.
Latz, E., Xiao, T.S., Stutz, A., 2013. Activation and regulation of the inflammasomes. Nat. Rev. Immunol. 13 (6), 397–411.
Lee, K.Y., DeMayo, F.J., 2004. Animal models of implantation. Reproduction 128 (6), 679–695.
Liao, Y., Tong, L., Tang, L., Wu, S., 2017. The role of cold-inducible RNA binding protein in cell stress response. Int. J. Cancer 141 (11), 2164–2173.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25 (4), 402–408.
Lunde, B.M., Moore, C., Varani, G., 2007. RNA-binding proteins: modular design for efficient function. Nat. Rev. Mol. Cell Biol. 8 (6), 479–490.
Nakamura, Y., Aihara, R., Iwata, H., Kuwayama, T., Shirasuna, K., 2021. IL1B triggers inflammatory cytokine production in bovine oviduct epithelial cells and induces neutrophil accumulation via CCL2. Am. J. Reprod. Immunol. 85 (5), 1–10.
Nguyen, L.T., Reverter, A., Cánovas, A., Venus, B., Anderson, S.T., Islas-Trejo, A., Dias, M.M., Crawford, N.F., Lehnert, S.A., Medrano, J.F., et al., 2018. STAT6, PBX2, and PBRM1 emerge as predicted regulators of 452 differentially expressed genes associated with puberty in Brahman heifers. Front. Genet. 9 (MAR), 1–13.
Nishiyama, H., Itoh, K., Kaneko, Y., Kishishita, M., Yoshida, O., Fujita, J., 1997. A Glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. J. Cell Biol. 137 (4), 899–908.
Okamura, H., Kashiwamura, S., Tsutsui, H., Yoshimoto, T., Nakanishi, K., 1998. Regulation of interferon-γ production by IL-12 and IL-18. Curr. Opin. Immunol. 10 (3), 259–264.
Park, H.J., Lee, S.J., Kim, S.H., Han, J., Bae, J., Kim, S.J., Park, C.G., Chun, T., 2011. IL-10 inhibits the starvation induced autophagy in macrophages via class I phosphatidylinositol 3-kinase (PI3K) pathway. Mol. Immunol. 48 (4), 720–727.
Pinheiro, J., Bates, D., DebRoy, S., Sarjar, D., Team, R.C., 2020. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3, pp. 1–145.
Pothmann, H., Flick, P., Tichy, A., Gabler, C., Drillich, M., 2021. Messenger RNA expression of selected factors at different sites of the bovine endometrium associated with uterine health. Front. Vet. Sci. 8 (March), 1–7.
Qiang, X., Yang, W.-L., Wu, R., Zhou, M., Jacob, A., Dong, W., Kuncewitch, M., Ji, Y., Yang, H., Wang, H., et al., 2013. Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nat. Med. 19 (11), 1489–1495.
R Core Team, 2019. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.
Rzechorzek, N.M., Connick, P., Patani, R., Selvaraj, B.T., Chandran, S., 2015. Hypothermic preconditioning of human cortical neurons requires Proteostatic priming. EBioMedicine 2 (6), 528–535.
Sadowski, H., Shuai, K., Darnell, J., Gilman, M., 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science (80-.) 261 (5129), 1739–1744.
Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., Yoshikawa, K., Akira, S., Takeda, J., 1999. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. EMBO J. 18 (17), 4657–4668.
Shirasuna, K., Karasawa, T., Takahashi, M., 2020. Role of the NLRP3 Inflammasome in preeclampsia. Front. Endocrinol. (Lausanne). 11 (February), 1–13.
Steffl, M., Schweiger, M., Sugiyama, T., Amselgruber, W.M., 2008. Review of apoptotic and non-apoptotic events in non-ciliated cells of the mammalian oviduct. Ann. Anat. 190 (1), 46–52.
Suzuki, T., Sakumoto, R., Hayashi, K.-G., Ogiso, T., Kunii, H., Shirozu, T., Kim, S.-W., Bai, H., Kawahara, M., Kimura, K., et al., 2018. Involvement of interferon-tau in the induction of apoptotic, pyroptotic, and autophagic cell death-related signaling pathways in the bovine uterine endometrium during early pregnancy. J. Reprod. Dev. 64 (6), 495–502.
Tong, G., Endersfelder, S., Rosenthal, L.-M., Wollersheim, S., Sauer, I.M., Bührer, C., Berger, F., Schmitt, K.R.L., 2013. Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices. Brain Res. 1504, 74–84.
Verhoeven, Y., Tilborghs, S., Jacobs, J., De Waele, J., Quatannens, D., Deben, C., Prenen, H., Pauwels, P., Trinh, X.B., Wouters, A., et al., 2020. The potential and controversy of targeting STAT family members in cancer. Semin. Cancer Biol. 60 (September 2019), 41–56.
Wagener, K., Gabler, C., Drillich, M., 2017. A review of the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows. Theriogenology 94, 21–30.
Wagener, K., Drillich, M., Aurich, C., Gabler, C., 2021. Endometrial inflammation at the time of insemination and its effect on subsequent fertility of dairy cows. Animals 11 (7).
Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science (80-.) 275 (5303), 1129–1132.
Yang, W.-L., Sharma, A., Wang, Z., Li, Z., Fan, J., Wang, P., 2016. Cold-inducible RNA-binding protein causes endothelial dysfunction via activation of Nlrp3 inflammasome. Sci. Rep. 6 (1), 26571.
Yockey, L.J., Iwasaki, A., 2018. Interferons and Proinflammatory cytokines in pregnancy and fetal development. Immunity 49 (3), 397–412.
Zhang, T., BaSang, W.D., Chang, W., Huo, S., Ma, X., Ju, X., Yu, S., Cui, S., 2021. Dynamics of apoptosis-related gene expression during follicular development in yak. J. Anim. Physiol. Anim. Nutr. (Berl). 105 (6), 1002–1013.
Zhong, P., Huang, H., 2017. Recent progress in the research of cold-inducible RNA-binding protein. Furture Sci. OA 3, FSO246.
Zhu, X., Bührer, C., Wellmann, S., 2016. Cold-inducible proteins CIRP and RBM3, a unique couple with activities far beyond the cold. Cell. Mol. Life Sci. 73 (20), 3839–3859.