Steroid hormones interact with natriuretic peptide C to delay nuclear maturation, to maintain oocyte–cumulus communication and to improve the quality of in vitro-produced embryos in cattle

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Short Title: Steroids and NPPC regulate oocyte maturation

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ABSTRACT

In vivo, oocyte maturation is triggered by the ovulatory LH surge while in vitro it is precociously induced when the cumulus oocyte complex (COC) is removed from the follicle. Natriuretic peptide type C (NPPC) delays germinal vesicle breakdown (GVBD) while increasing oocyte-cumulus communication during in vitro maturation (IVM) in cattle. We first tested the hypothesis that steroids secreted by the follicle (oestradiol-17β, progesterone and androstenedione) interact with NPPC to delay GVBD and to maintain oocyte-cumulus communication. Then we assessed the effects of steroid hormones, NPPC and the combination of both in a pre-IVM culture on embryo production. The combination of NPPC with steroids delayed GVDB and increased NPR2 mRNA abundance in cumulus cells during culture, and maintained oocyte-cumulus communication at levels not different from non-cultured controls. The addition of steroids and/or NPPC to a pre-IVM culture did not alter blastocyst rates after IVF, but supplementation with steroids increased blastocyst total cell number. This study provides evidence for the first time in cattle that steroids interact with NPPC to regulate oocyte nuclear maturation and oocyte-cumulus communication, and improve oocyte developmental competence.

Keywords: steroid hormone, oocyte, cumulus cell, in vitro maturation.

1 INTRODUCTION

Oocyte maturation is a critical step for in vitro embryo production (IVP) and its efficiency is compromised by culture systems that do not adequately replace
physiological conditions (Rizos et al. 2002; Farin et al. 2007). In vivo, intrafollicular factors maintain meiotic arrest and the oocyte remains at the germinal vesicle (GV) stage until the preovulatory LH surge triggers the secretion of the epidermal growth factor (EGF)-like peptides amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) from granulosa cells, which then induce meiosis resumption and cumulus expansion (reviewed by Gilchrist 2011). However, when the oocyte is removed from the follicle before the LH surge, spontaneous resumption of meiosis occurs (Pincus and Enzmann 1935) before the completion of cytoplasmic maturation, and this compromises developmental competence (Sánchez and Smitz 2012). Strategies to maintain meiotic arrest before initiating in vitro maturation (IVM) may improve embryo development.

Oocyte development relies on bidirectional communication with cumulus cells mediated by secreted factors and by transzonal cytoplasmic projections (TZP), at the end of which gap junctions (GJ) allow the transport of ions, metabolites, amino acids, RNA and other small regulatory molecules (Albertini et al. 2001; Eppig 2001; Macaulay et al. 2014). In the cumulus-oocyte complex (COC), GJ are composed mainly of connexin 43 (Cx43) (Grazul-Bilska et al. 1997). These junctions remain open until the LH-induced release of EGF-like peptides leads to Cx43 phosphorylation via a mitogen activated protein kinase (MAPK) pathway (Park et al. 2004). The closure of GJ prevents the transfer of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) from cumulus cells to the oocyte, altering phosphodiesterase 3 (PDE3) activity to further decrease cAMP levels. This causes the release of maturation-promoting factor (MPF) and the resumption of meiosis (reviewed by Gilchrist et al. 2016). Recent data from studies with cattle also indicate that GJ-mediated cell
communication within the COC regulates chromatin remodeling and transcriptional activity of the oocyte, and thus is of crucial importance for oocyte developmental competence (Luciano et al. 2014). Specifically, it has been shown that GJ must remain open to allow gradual chromatin remodeling *in vitro*, while premature GJ interruption leads to abrupt chromatin condensation (Luciano et al. 2011; Lodde et al. 2013).

Another important regulator of meiotic arrest is natriuretic peptide type C (NPPC), which is predominantly produced by mural granulosa cells and inhibits germinal vesicle breakdown (GVBD) in mice (Zhang et al. 2010) and in cattle (Franciosi et al. 2014). In mice, NPPC activity in cumulus cells is regulated by steroids; oestradiol stimulates the ability of NPPC to inhibit GVBD, and both oestradiol and testosterone increase natriuretic peptide receptor 2 (NPR2) mRNA levels (Zhang et al. 2011). It is not known if steroids play a role in the NPPC-mediated maintenance of meiotic arrest in cattle.

The hypothesis of the present study was that NPPC interacts with steroids, at approximately the same concentrations found in healthy dominant follicles (Ireland and Roche 1983; Beg et al. 2002), to improve *in vitro* embryo production in cattle. The specific objectives were to test the effects of a combination of steroid hormones and NPPC on GVBD dynamics, oocyte-cumulus cells GJ-mediated communication, *NPR2* mRNA abundance in cumulus cells, and finally on embryo development after in vitro fertilization (IVF).

2 MATERIALS AND METHODS

2.1 Chemicals
Chemicals and reagents were purchased from Sigma-Aldrich Brasil Ltda (Sao Paulo, Brazil) or Sigma-Aldrich S.r.l. (Milan, Italy) unless otherwise mentioned. Recombinant human FSH (Puregon) was obtained from Schering-Plough (Sao Paulo, Brazil).

2.2 Ovaries and COC collection

Ovaries of adult Nellore (Bos indicus), Angus (Bos taurus) and crossbred cows were obtained at an abattoir near Sao Paulo State University – Campus of Botucatu (Brazil), and ovaries from Holstein cows (Bos taurus) were obtained at an abattoir near the University of Milan (INALCA Spa, Ospedaletto Lodigiano, LO, IT 2270M CE, Italy). Ovaries transported to the laboratories in sterile saline solution (0.9% NaCl) at 26-28°C. COC were aspirated from follicles of 2 to 6mm in diameter with a 19-gauge needle and pooled in a 15mL conical tube. After sedimentation, COC were recovered and selected with a stereomicroscope. Only COC with homogeneous or slightly granulated cytoplasm and at least 5 compact layers of cumulus cells were used in these experiments. COC were washed two times in TCM199 with Earle’s salts and 25mM Hepes, supplemented with 75μg/mL amikacin and 4mg/mL BSA (M199D), and groups of 20 oocytes were cultured according to the experimental design (see below) in four-well dishes at 38.5°C and 5% CO₂ in humidified air.

2.3 Assessment of oocyte-cumulus GJ-mediated communication

Intercellular communication between the oocyte and cumulus cells was assessed as previously described (Luciano et al. 2004). Briefly, a 3% solution of Lucifer Yellow (LY) in 5mM lithium chloride was pressure injected into the oocyte,
and the spread of the dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope (Nikon Diaphot; Nikon Corp.) 10 min after the injection. Obvious transfer of dye to the cumulus denoted functional GJ-mediated cell communication, and the percentage of COC within each group with functional cell communication was calculated.

### 2.4 Assessment of germinal vesicle status

Germinal vesicle status was examined by fluorescence microscopy after mechanical denudation and fixation of the oocytes in 500µL Dulbecco PBS (DPBS) with 60% methanol for 30 min at 4°C, followed by staining with 1µg/mL of Hoechst 33342. Oocytes were classified as in germinal vesical (GV) or germinal vesicle breakdown (GVBD) depending on the integrity of the germinal vesicle. Oocytes with intact germinal vesicle were classified as GV oocytes and those with irregular/partly degrading or absent germinal vesicle as GVBD oocytes.

### 2.5 In vitro fertilization (IVF), embryo culture and assessment of embryo total cell number

IVF and embryo culture was performed as previously described (Lodde et al. 2007). The content of a straw of cryopreserved bovine semen (only one batch from the same Bull was used throughout the study) was thawed and spermatozoa were separated on a 45–90% Percoll gradient. Spermatozoa were counted and diluted to the final concentration of 2 x 10^6 spermatozoa/ml in a modified Tyrode solution supplemented with 0.6% BSA (fatty acid-free), 10µg/mL heparin, 20µM penicillamine, 1µM epinephrine, and 100µM hypotaurine. Cumulus-oocyte
complexes and sperm cells were incubated for 18 hours at 38.58°C under 5% CO2 in humidified air. Presumptive zygotes were then washed, and cumulus cells removed by vortexing for 1 min in 500 µL of a modified synthetic oviduct fluid supplemented with 0.3% BSA fraction V (fatty acid-free), MEM essential and nonessential amino acids, 0.72 mM sodium pyruvate, and buffered with 10 mM HEPES and 5 mM NaHCO3. Presumptive zygotes were then transferred to the embryo culture medium, which was synthetic oviduct fluid buffered with 25 mM NaHCO3 and supplemented with MEM essential and nonessential amino acids, 0.72 mM of sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate, and 5% bovine calf serum. Incubation was performed at 38.58°C under 5% CO2, 5% O2, and 90% N2 in humidified air for 8 days. Blastocysts were counted and morphologically classified as not expanded, expanded or hatched under a stereomicroscope nine days after fertilization (Jakobsen et al. 2006). The embryos were then fixed in 60% methanol in DPBS, stained with 1 µg/mL of Hoechst 33342, and cell nuclei were counted under a fluorescence microscope.

2.6 Gene expression analysis

Total RNA was extracted from cumulus cells mechanically isolated from groups of 15-20 COCs subjected to the pre-IVM treatments described above using the RNeasy® kit as recommended by the manufacturer. After purification, RNA samples were diluted in 30 µL of RNase free water. Total RNA concentrations were measured by spectrophotometry using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA), and purity verified by measuring the A260:A280 ratio. Total RNA (100 ng/reaction) was incubated with DNase (1 U/µg; Invitrogen, Sao Paulo, Brazil) and then reverse transcribed using Oligo-dT
primers and Omniscript (Qiagen, Mississauga, ON, CA). Samples were incubated at 37°C for 60 minutes and then at 93°C for 3 minutes for enzyme inactivation. Relative RT-qPCR analysis was performed with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, Sao Paulo, Brazil). The final volume of the PCR mix was 24 µL and 1 µL of cDNA sample, thermocycling conditions were: 95 °C for 10 minutes (1 cycle), denaturing at 95°C for 10 seconds, followed by annealing for 1 minute (40 cycles). The reference gene was CYC-A as previously validated in our laboratory by testing several candidate genes (Machado et al. 2009; Caixeta et al. 2013). Bovine-specific primers to amplify NPR2 were designed with the PrimerQuest Tool (NM_174126.2). The PCR primer sequences were as follows: CYC-A, 5’-GCCATGGAGCGCTTTGG3’ (forward) and 5’-CCACAGTCAGCAATGGTGATCT3’ (reverse); and NPR2 5’ATGACAGCATCAACCTGGACTGGA3’ (forward) and 5’AGCAGAAACGACTATCCACCACA3’ (reverse). Melting curve analysis indicated amplification of a single amplicon. Each sample was assayed in duplicate, and the relative expression values for each gene were calculated using the ΔΔCq method with efficiency correction and using one control sample as calibrator (Pfaffl 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers et al. 2003).

2.7 Statistical Analysis

The data in the form of percentages were arcsine transformed. In order to reach normal distribution, mRNA relative values were log transformed. Variance
homogeneity within treatments was confirmed before testing the effects of treatments. The effects of treatments on the percentage of GV/GVBD oocytes, percentage of COCs with functional oocyte-cumulus communication, $NPR2$ mRNA abundance in cumulus cells, embryo production rates and embryo cell number were tested by analysis of variance (ANOVA). When ANOVA indicated a significant effect of treatment, means were compared with the Tukey-Kramer HSD test. These analyses were performed with the JMP software (SAS Institute, Cary, NC, USA), and differences were considered significant when $P<0.05$.

2.8 Experimental design

To measure the effects of steroids and NPPC, separately or together, on GVBD rates and abundance of $NPR2$ mRNA in cumulus cells, groups of 20 Nellore COCs were cultured for 9 hours in 400µL of pre-IVM medium alone (Treatment ‘CON’; TCM 199 containing Earle’s salts, L-glutamine and NaHCO3, and supplemented with 0.4% fatty acid-free BSA, 22µg/mL sodium pyruvate and 75µg/mL amikacin), pre-IVM medium supplemented with 100nM NPPC (Treatment ‘NPPC’), pre-IVM medium supplemented with steroids and FSH (Treatment ‘FS’; 500ng/mL oestradiol-17β, 50ng/mL progesterone, 50ng/mL androstenedione and 10⁻⁴IU/mL FSH), or in pre-IVM medium supplemented with steroids, FSH and NPPC (Treatment ‘FS+NPPC’). Steroid hormones were added at concentrations found in the follicular fluid of growing dominant follicles (Ireland and Roche 1983; Kaneko et al. 1991; Beg et al. 2002). The concentration of NPPC chosen is the lowest dose that maintained bovine oocytes in GV stage in a previous study (Franciosi et al. 2014). An additional group of COC was included in each replicate that was not cultured and served as a time 0h control for both
endpoints (Treatment ‘CON-0h’). At the end of culture, oocytes were mechanically separated from cumulus cells by repeated pipetting in PBS without calcium and magnesium, and fixed and stained to assess germinal vesicle status as described above. Cumulus cells were transferred to 1.5mL tubes, collected by centrifugation for 5 min at 700g and frozen at -80°C in 350μL of RNA extraction lysis buffer (RNeasy® kit; Qiagen, Mississauga, ON, Canada) to assess the effects of treatments on the abundance of NPR2 mRNA. These experiments were performed four times.

The effects of steroids and NPPC, separately or together on GV status and oocyte-cumulus cells GJ-mediated communication were tested in groups of 20 oocytes from Holstein cows cultured in CON, NPPC, FS and FS+NPPC media as described above. In the first experiment, at the end of culture, oocytes were isolated and fixed to assess germinal vesicle status. In a second experiment, intact COCs were used to assess effects of treatments on functional oocyte-cumulus cells GJ-mediated communication as described above. An additional group of COC was included in each replicate that was not cultured and served as a time 0h control for both endpoints (Treatment ‘CON-0h’). Both experiments above were performed on three independent replicates.

Finally, to assess the effects of steroids and NPPC in a pre-IVM culture step on in vitro embryo production, groups of 20 oocytes from Nellore, Angus or crossbred cows were cultured in CON, NPPC, FS and FS+NPPC media as described above for 9 hours, and then subjected to IVM in 400μL IVM medium containing 10ng/mL insulin-like grown factor 1 (IGF-1), 100ng/mL AREG, 10^-2 IU/mL FSH and concentrations of oestradiol-17β (50ng/mL) and progesterone (150ng/mL) observed in bovine follicles after the LH surge (Fortune and Hansel
1985; Komar et al. 2001). After 24h, oocytes underwent *in vitro* fertilization (IVF) as described above. This experiment was replicated four times and in each replicate COCs of a single breed were used; Nellore and crossbred COCs were used in one replicate each, and Angus COCs were used in two replicates. The numbers of COCs subjected to each treatment were 195, 196, 197 and 195 for CON, NPPC, FS and FS+NPPC, respectively.

3 RESULTS

Culture of COCs in pre-IVM medium without additives significantly reduced the proportion of oocytes in GV stage in Nellore cattle compared with uncultured controls (Fig 1a). The addition of steroids+FSH or of NPPC caused a numerical but non-significant increase in the proportion of oocytes remaining in GV, but when added together they significantly increased the proportion of GV arrested oocytes to levels observed in the non-cultured controls (Fig. 1a).

Abundance of *NPR2* mRNA in cumulus cells was not altered by 9 h culture in pre-IVM medium, and while neither NPPC nor steroids+FSH altered *NPR2* mRNA levels, the combination of both treatments significantly increased *NPR2* mRNA levels above those observed in non-cultured controls (Fig 1b).

Culture of *Bos taurus* COC in pre-IVM medium significantly reduced the proportion of oocytes in GV arrest compared with non-cultured controls (Fig 2a), and supplementation with NPPC but not steroids+FSH significantly increased the proportion of GV-arrested oocytes. The combination of NPPC and steroids+FSH was not different from NPPC alone. Similarly, culture in pre-IVM medium significantly reduced the percentage of COCs with functional oocyte-cumulus
cells GJ-mediated communication (Fig. 2b), and supplementation with NPPC but
not FSH+steroids significantly increased the percentage of COCs with open
oocyte-cumulus cells GJ-mediated communication. The combination of NPPC
with steroids+FSH was not different from NPPC alone. However, the combination
of NPPC with steroids+FSH, but not NPPC alone, promoted GV arrest and
oocyte-cumulus cells GJ-mediated communication at levels not different from
non-cultured controls (Fig.2ab).

When pre-IVM was followed by IVM and IVF, supplementation of the pre-
IVM medium with NPPC, steroids+FSH or both treatments combined did not alter
blastocyst production rates nor the percentage of hatched and expanded
blastocysts produced (Fig. 3ab). Addition of steroids+FSH but not of NPPC to the
pre-IVM medium increased blastocyst total cell number in comparison with base
medium without additives. Supplementation of steroids+FSH was not different
from supplementation with NPPC. The combination of steroids+FSH with NPPC
during pre-IVM elicited the highest blastocyst cell number, which was not
statistically different from steroids+FSH, but was greater than that provided by
NPPC alone (Fig. 3c).

4 DISCUSSION

Previous studies have shown that NPPC inhibits oocyte nuclear
maturation in several species including the mouse, cow, pig and cat (Zhang et al.
2010; Franciosi et al. 2014; Blaha et al. 2015; Zhong et al. 2015). Studies in mice
suggest that oestradiol-17β increases the sensitivity of cumulus cells to NPPC
and is required for NPPC to inhibit meiotic resumption (Zhang et al. 2011). There
are no previous studies exploring the potential interaction of NPPC and a combination of steroid hormones at physiological levels in the regulation of oocyte nuclear maturation. Here we show novel evidence that the presence of oestradiol-17β, progesterone and androstenedione enhances the ability of NPPC to delay germinal vesicle breakdown and to maintain oocyte-cumulus cells GJ-mediated communication in the bovine COC by mechanisms involving an increase in the sensitivity of cumulus cells to NPPC. In addition, we demonstrate that supplementation of the culture medium with physiological concentrations of steroids in a pre-IVM culture step improves embryo quality after IVF.

Pre-IVM cultures with phosphodiesterase inhibitors or NPPC can prolong meiotic arrest and improve embryonic developmental competence in cattle (Albuz et al. 2010; Franciosi et al. 2014). A previous study in mice demonstrated that both oestradiol-17β and testosterone individually inhibit germinal vesicle breakdown, while increasing the expression of NPR2 in cumulus cells. In the same study, oestradiol-17β was required to sustain the ability of NPPC to stimulate cGMP production during culture (Zhang et al. 2011). Differently than in mice, oestradiol-17β did not synergize with NPPC to delay GVBD in cattle (Franciosi et al. 2014). Nevertheless, data from the present study suggest that the presence of all three major steroid hormones at approximately intrafollicular concentrations enhances the ability of NPPC to delay GVBD and to prolong oocyte-cumulus cells GJ-mediated communication in the cow. This suggests a physiological role for intrafollicular steroids in the regulation of oocyte nuclear maturation through NPPC signaling in cattle, although oestradiol-17β alone may not be as effective as in mice to sensitize bovine cumulus cells to NPPC (Zhang et al. 2011; Franciosi et al. 2014).
Interestingly, in the present study the combination of steroids+FSH and NPPC induced a two-fold increase in NPR2 mRNA abundance in cumulus cells compared with treatment with NPPC alone. Although we have not measured NPR2 protein or activity, we speculate that the synergistic effect of steroids and NPPC in cattle is at least in part a consequence of increased sensitivity of cumulus cells to NPPC. This is consistent with previous data in mice, in which induction of greater NPR2 mRNA abundance was associated with higher NPR2 protein expression and cGMP production by cumulus cells (Zhang et al. 2011).

The inhibitory effect of NPPC on germinal vesicle breakdown was associated with prolonged oocyte-cumulus cells GJ-mediated communication in cattle before (Franciosi et al. 2014). In this study, steroid hormones enhanced the effects of NPPC on oocyte-cumulus cells GJ-mediated communication in the bovine COC. A more efficient or prolonged communication would allow greater transfer of cGMP from cumulus cells to the oocyte, decreasing PDE3 activity on cAMP and thus inhibiting meiotic resumption (Zhang et al. 2010). Therefore, in addition to the stimulation of NPPC signaling and cGMP production in cumulus cells, the increased flow of cGMP to the oocyte is per se a plausible mechanism by which steroids and NPPC combined delayed oocyte nuclear maturation.

In this study, we aimed for a more physiological system capable of sustaining meiotic arrest and oocyte-cumulus cells GJ-mediated communication, therefore steroid hormones were added to the pre-IVM medium at concentrations observed in the follicular fluid of growing dominant follicles (Ireland and Roche 1983; Kaneko et al. 1991; Beg et al. 2002). Our data further support that the administration of low concentrations of FSH appear to be effective in promoting intercellular communication within the cumulus-oocyte complex probably by
regulating Cx43 phosphorylation in a way that favors GJ coupling (Atif et al. 2005; Luciano et al. 2011; El-Hayek and Clarke 2015).

During final antral follicle growth, oestradiol-17\(\beta\) levels are high and progesterone levels are low, but around the time of ovulation the LH surge triggers the differentiation of theca and granulosa cells to small and large luteal cells, and steroidogenesis is directed towards progesterone production (Fortune and Hansel 1985; Komar et al. 2001). Higher concentrations of progesterone and lower concentrations of oestradiol-17\(\beta\) in the preovulatory follicle were associated with better cumulus expansion and blastocyst production (Aardema et al. 2013). Moreover, progesterone signaling during IVM is necessary for cumulus expansion and oocyte developmental competence (Aparicio et al. 2011). These observations led us to increase progesterone concentration and to decrease oestradiol-17\(\beta\) concentration in the IVM medium. Also aiming for a more physiological induction of oocyte maturation, IVM was performed in the presence of AREG and FSH at concentrations lower than the supraphysiological levels commonly used (Richani et al. 2014; Sugimura et al. 2014). Supplementation of the pre-IVM culture medium with NPPC and steroids, separately or combined, did not affect blastocyst rates in the present study. On the other hand, supplementation of the pre-IVM medium with steroids+FSH or steroids+FSH combined with NPPC increased blastocyst total cell number, the greatest numerical increase being observed with the combination. Since supplementation of the pre-IVM medium with steroids+FSH did not significantly affect GVBD or oocyte-cumulus cells GJ-mediated communication in the present study, the beneficial effect of steroids on developmental competence appear to involve other processes in cumulus cells. As mentioned above, progesterone signaling...
stimulates cumulus expansion in cattle and is crucial for developmental competence (Aparicio et al. 2011). In mice, oestrogens increase transcription of a variety of genes regulating expansion, metabolism, proliferation and EGF-stimulated pathways in cumulus cells (Sugiura et al. 2010; Emori et al. 2013). We are not aware of any studies assessing the effects of different steroids combined on cumulus cell function.

Data from the present study suggest that steroid hormones interact with NPPC to inhibit germinal vesicle breakdown in both Bos indicus and Bos taurus. However, our results also suggest that Bos taurus oocytes are more susceptible to meiotic resumption once the COC is removed from the follicle. In fact, although we cannot directly compare GV rates observed in Bos taurus and Bos indicus, around 68% of the Bos indicus oocytes cultured in base medium for 9 hours remained at GV stage, whereas only 38% of the Bos taurus oocytes subjected to the same culture conditions remained at GV (Fig. 1 and 2). This finding is possibly related to differences in endocrine profiles and follicular dynamics between these subspecies, and to the lower performance of oocytes from Bos taurus compared to Bos indicus in IVM/IVF (reviewed by Sartori et al. 2016).

In conclusion, the present study provides evidence for the first time that steroids at intrafollicular levels interact with NPPC to delay meiotic resumption and to prolong oocyte-cumulus cells GJ-mediated communication in cattle. Moreover, our data suggest that physiological exposure to steroids may be important to optimize developmental competence of the COC. Taken together, these data point to physiological roles for steroids and NPPC in the regulation of oocyte maturation and provide valuable references for the improvement of IVM/IVF outcomes in cattle.
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REFERENCES

Aardema, H., Roelen, B.A., van Tol, H.T., Oei, C.H., Gadella, B.M., and Vos, P.L. (2013) Follicular 17β-estradiol and progesterone concentrations and degree of cumulus cell expansion as predictors of in vivo-matured oocyte developmental competence in superstimulated heifers. *Theriogenology* **80**, 576-83

Albertini, D.F., Combelles, C.M., Benecchi, E., and Carabatsos, M.J. (2001) Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* **121**, 647-53

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G., and Gilchrist, R.B. (2010) Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod* **25**, 2999-3011

Aparicio, I.M., García-Herreros, M., O'Shea, L.C., Hensey, C., Lonergan, P., and Fair, T. (2011) Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. *Biol Reprod* **84**, 910-21

Atef, A., François, P., Christian, V., and Marc-André, S. (2005) The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. *Mol Reprod Dev* **71**, 358-67

Beg, M.A., Bergfelt, D.R., Kot, K., and Ginther, O.J. (2002) Follicle selection in cattle: dynamics of follicular fluid factors during development of follicle dominance. *Biol Reprod* **66**, 120-6
Blaha, M., Nemcova, L., and Prochazka, R. (2015) Cyclic guanosine monophosphate does not inhibit gonadotropin-induced activation of mitogen-activated protein kinase 3/1 in pig cumulus-oocyte complexes. Reprod Biol Endocrinol 13, 1

Caixeta, E.S., Sutton-McDowall, M.L., Gilchrist, R.B., Thompson, J.G., Price, C.A., Machado, M.F., Lima, P.F., and Buratini, J. (2013) Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. Reproduction 146, 27-35

Caixeta, E.S., Sutton-McDowall, M.L., Gilchrist, R.B., Thompson, J.G., Price, C.A., Machado, M.F., Lima, P.F., and Buratini, J. (2013) Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. Reproduction 146, 27-35

El-Hayek, S., and Clarke, H.J. (2015) Follicle-Stimulating Hormone Increases Gap Junctional Communication Between Somatic and Germ-Line Follicular Compartments During Murine Oogenesis. Biol Reprod 93, 47

Emori, C., Wigglesworth, K., Fujii, W., Naito, K., Eppig, J.J., and Sugiura, K. (2013) Cooperative effects of 17β-estradiol and oocyte-derived paracrine factors on the transcriptome of mouse cumulus cells. Endocrinology 154, 4859-72

Eppig, J.J. (2001) Oocyte control of ovarian follicular development and function in mammals. Reproduction 122, 829-38

Farin, C.E., Rodriguez, K.F., Alexander, J.E., Hockney, J.E., Herrick, J.R., and Kennedy-Stoskopf, S. (2007) The role of transcription in EGF- and FSH-mediated oocyte maturation in vitro. Anim Reprod Sci 98, 97-112

Fortune, J.E., and Hansel, W. (1985) Concentrations of steroids and gonadotropins in follicular fluid from normal heifers and heifers primed for superovulation. Biol Reprod 32, 1069-79

Franciosi, F., Coticchio, G., Lodde, V., Tessaro, I., Modina, S.C., Fadini, R., Dal Canto, M., Renzini, M.M., Albertini, D.F., and Luciano, A.M. (2014) Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. Biol Reprod 91, 61

Gilchrist, R.B. (2011) Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. Reprod Fertil Dev 23, 23-31

Gilchrist, R.B., Luciano, A.M., Richani, D., Zeng, H., Wang, X., De Vos, M., Sugimura, S., Smitz, J., Richard, F.J., and Thompson, J.G. (2016) Oocyte maturation and quality: role of cyclic nucleotides. Reproduction 152, R143-57

Grazul-Bilska, A.T., Reynolds, L.P., and Redmer, D.A. (1997) Gap junctions in the ovaries. Biol Reprod 57, 947-57

18
Ireland, J.J., and Roche, J.F. (1983) Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: changes in concentration of hormones in follicular fluid and specific binding of gonadotropins to follicles. *J Anim Sci* **57**, 157-67

Jakobsen, A.S., Thomsen, P.D., and Avery, B. (2006) Few polyploid blastomeres in morphologically superior bovine embryos produced in vitro. *Theriogenology* **65**, 870-81

Kaneko, H., Terada, T., Taya, K., Watanabe, G., Sasamoto, S., Hasegawa, Y., and Igarashi, M. (1991) Ovarian follicular dynamics and concentrations of oestradiol-17 beta, progesterone, luteinizing hormone and follicle stimulating hormone during the periovulatory phase of the oestrous cycle in the cow. *Reprod Fertil Dev* **3**, 529-35

Komar, C.M., Berndtson, A.K., Evans, A.C., and Fortune, J.E. (2001) Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. *Biol Reprod* **64**, 1797-805

Lodde, V., Franciosi, F., Tessaro, I., Modina, S.C., and Luciano, A.M. (2013) Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. *J Assist Reprod Genet* **30**, 1219-26

Lodde, V., Modina, S., Galbusera, C., Franciosi, F., and Luciano, A.M. (2007) Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence. *Mol Reprod Dev* **74**, 740-9

Luciano, A.M., Franciosi, F., Dieci, C., and Lodde, V. (2014) Changes in large-scale chromatin structure and function during oogenesis: a journey in company with follicular cells. *Anim Reprod Sci* **149**, 3-10

Luciano, A.M., Franciosi, F., Modina, S.C., and Lodde, V. (2011) Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s). *Biol Reprod* **85**, 1252-9

Luciano, A.M., Modina, S., Vassena, R., Milanesi, E., Lauria, A., and Gandolfi, F. (2004) Role of intracellular cyclic adenosine 3′,5′-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. *Biol Reprod* **70**, 465-72

Macaulay, A.D., Gilbert, I., Caballero, J., Barreto, R., Fournier, E., Tossou, P., Sirard, M.A., Clarke, H.J., Khandjian, É., Richard, F.J., Hyttel, P., and Robert, C. (2014) The gametic synapse: RNA transfer to the bovine oocyte. *Biol Reprod* **91**, 90

Machado, M.F., Portela, V.M., Price, C.A., Costa, I.B., Ripamonte, P., Amorim, R.L., and Buratini, J. (2009) Regulation and action of fibroblast growth factor 17 in bovine follicles. *J Endocrinol* **202**, 347-53
Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L., and Conti, M. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* **303**, 682-4

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45

Pincus, G., and Enzmann, E.V. (1935) THE COMPARATIVE BEHAVIOR OF MAMMALIAN EGGS IN VIVO AND IN VITRO: I. THE ACTIVATION OF OVARIAN EGGS. *J Exp Med* **62**, 665-75

Ramakers, C., Ruijter, J.M., Deprez, R.H., and Moorman, A.F. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**, 62-6

Richani, D., Wang, X., Zeng, H.T., Smitz, J., Thompson, J.G., and Gilchrist, R.B. (2014) Prematuration with cAMP modulators in conjunction with EGF-like peptides during in vitro maturation enhances mouse oocyte developmental competence. *Mol Reprod Dev* **81**, 422-35

Rizos, D., Ward, F., Duffy, P., Boland, M.P., and Lonergan, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* **61**, 234-48

Sartori, R., Gimenes, L.U., Monteiro, P.L., Melo, L.F., Baruselli, P.S., and Bastos, M.R. (2016) Metabolic and endocrine differences between Bos taurus and Bos indicus females that impact the interaction of nutrition with reproduction. *Theriogenology* **86**, 32-40

Sugimura, S., Ritter, L.J., Sutton-McDowall, M.L., Mottershead, D.G., Thompson, J.G., and Gilchrist, R.B. (2014) Amphiregulin co-operates with bone morphogenetic protein 15 to increase bovine oocyte developmental competence: effects on gap junction-mediated metabolite supply. *Mol Hum Reprod* **20**, 499-513

Sugiuara, K., Su, Y.Q., Li, Q., Wigglesworth, K., Matzuk, M.M., and Eppig, J.J. (2010) Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. *Mol Endocrinol* **24**, 2303-14

Sánchez, F., and Smitz, J. (2012) Molecular control of oogenesis. *Biochim Biophys Acta* **1822**, 1896-912

Zhang, M., Su, Y.Q., Sugiuara, K., Wigglesworth, K., Xia, G., and Eppig, J.J. (2011) Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. *Endocrinology* **152**, 4377-85

Zhang, M., Su, Y.Q., Sugiuara, K., Xia, G., and Eppig, J.J. (2010) Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* **330**, 366-9
Zhong, Y., Lin, J., Liu, X., Hou, J., Zhang, Y., and Zhao, X. (2015) C-Type natriuretic peptide maintains domestic cat oocytes in meiotic arrest. *Reprod Fertil Dev* **28**, 1553–1559

**FIGURES**

Figure 1. Effects of steroid hormones and NPPC on (a) the percentage of GV-arrested oocytes and (b) NPR2 mRNA abundance in cumulus cells after culture for 9 hours. *Bos indicus* COCs were cultured with treatments CON (base medium only), NPPC (base medium + NPPC), FS (base medium + steroids/FSH) and FS+NPPC (base medium + steroids/FSH + NPPC). Non-cultured COCs were included in the analyses as a time 0h control (CON-0h). Experiments were performed on 4 independent replicates with 15-20 oocytes per treatment). Different letters indicate statistically significant differences (P<0.05).
Figure 2. Effects of steroid hormones and NPPC on the percentage of GV-arrested oocytes (a), and percentage of COCs with functional oocyte-cumulus communication (b) after culture for 9 hours. *Bos taurus* COCs were cultured with treatments CON (base medium only), NPPC (base medium + NPPC), FS (base medium + steroids/FSH) and FS+NPPC (base medium + steroids/FSH + NPPC). Non-cultured COCs were included in the analyses as a time 0h control (CON-0h). Experiments were performed in 3 independent replicates with 15-20 oocytes per treatment. Different letters indicate statistically significant differences (P<0.05). (c) Representative images showing COCs with open or closed GJ oocyte-cumulus communication as detected by transfer of Lucifer Yellow between the oocyte and cumulus cells.
Figure 3. Effects of steroid hormones and NPPC added separately or together in a pre-IVM culture on embryo production as measured by the percentage of blastocysts in relation to total oocytes (a), percentage of expanded and hatched blastocysts in relation to total blastocysts (b) and blastocyst cell number (c). Bovine COCs were cultured for 9 hours with pre-IVM treatments CON (base medium), NPPC (base medium+NPPC), FS (base medium+steroids/FSH) and FS+NPPC (base medium+steroids/FSH+NPPC), followed by IVM, IVF and embryo culture for 8 days. This experiment was replicated 4 times with 195, 196, 197 and 195 COCs allocated to treatments CON, NPPC, FS and FS+NPPC, respectively. Different letters indicate statistically significant differences (P <0.05).