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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

Ana Caroline S. Soares ^A, Valentina Lodde ^B, Rodrigo G. Barros ^A, Christopher A. Price ^C, Alberto M. Luciano ^B and José Buratini ^{A D}

+ Author Affiliations

^A Ovarian Molecular Physiology Laboratory, Department of Physiology, Institute of Biosciences, Sao Paulo State University, Botucatu, Sao Paulo, 18618-970, Brazil.

^B Reproductive and Developmental Biology Laboratory, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria, 10 - 20133, Milan, Italy.

^C Centre de recherche en reproduction et fertilité, Faculté de médecine vétérinaire, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec J2S 7C6, Canada.

^D Corresponding author. Email: buratini@ibb.unesp.br

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1 **Title: Steroid hormones interact with natriuretic peptide type C to delay**
2 **nuclear maturation, to maintain oocyte-cumulus communication and to**
3 **improve quality of *in vitro* produced embryos in cattle**

4
5 **Short Title: Steroids and NPPC regulate oocyte maturation**
6

7 Ana Caroline S. Soares¹; Valentina Lodde²; Rodrigo G. Barros¹; Christopher A.
8 Price³; Alberto M. Luciano²; José Buratini¹

9

10 ¹Ovarian Molecular Physiology Laboratory, Department of Physiology, Institute
11 of Biosciences, Sao Paulo State University, São Paulo, Brazil.

12

13 ²Reproductive and Developmental Biology Laboratory, Department of Health,
14 Animal Science and Food Safety, University of Milan, Milan, Italy.

15

16 ³Research Center of the Faculty of Veterinary Medicine, Department of
17 Veterinary Biomedicine, University of Montreal, St-Hyacinthe, Quebec, Canada.

18

19 Correspondence:

20 José Buratini

21 Departamento de Fisiologia, IB, Universidade Estadual Paulista

22 Rubião Junior, Botucatu, SP, Brazil, 18618-970

23 Telephone/Fax: (55) 14.38800330

24 E-mail: buratini@ibb.unesp.br

25 **ABSTRACT**

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

43

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

45

46 **1 INTRODUCTION**

47

48 Oocyte maturation is a critical step for *in vitro* embryo production (IVP) and
49 its efficiency is compromised by culture systems that do not adequately replace

50 physiological conditions (Rizos *et al.* 2002; Farin *et al.* 2007). *In vivo*,
51 intrafollicular factors maintain meiotic arrest and the oocyte remains at the
52 germinal vesicle (GV) stage until the preovulatory LH surge triggers the secretion
53 of the epidermal growth factor (EGF)-like peptides amphiregulin (AREG),
54 epiregulin (EREG) and betacellulin (BTC) from granulosa cells, which then induce
55 meiosis resumption and cumulus expansion (reviewed by Gilchrist 2011).
56 However, when the oocyte is removed from the follicle before the LH surge,
57 spontaneous resumption of meiosis occurs (Pincus and Enzmann 1935) before
58 the completion of cytoplasmic maturation, and this compromises developmental
59 competence (Sánchez and Smitz 2012). Strategies to maintain meiotic arrest
60 before initiating *in vitro* maturation (IVM) may improve embryo development.

61 Oocyte development relies on bidirectional communication with cumulus
62 cells mediated by secreted factors and by transzonal cytoplasmic projections
63 (TZP), at the end of which gap junctions (GJ) allow the transport of ions,
64 metabolites, amino acids, RNA and other small regulatory molecules (Albertini *et al.*
65 *et al.* 2001; Eppig 2001; Macaulay *et al.* 2014). In the cumulus-oocyte complex
66 (COC), GJ are composed mainly of connexin 43 (Cx43) (Grazul-Bilska *et al.*
67 1997). These junctions remain open until the LH-induced release of EGF-like
68 peptides leads to Cx43 phosphorylation via a mitogen activated protein kinase
69 (MAPK) pathway (Park *et al.* 2004). The closure of GJ prevents the transfer of
70 cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate
71 (cAMP) from cumulus cells to the oocyte, altering phosphodiesterase 3 (PDE3)
72 activity to further decrease cAMP levels. This causes the release of maturation-
73 promoting factor (MPF) and the resumption of meiosis (reviewed by Gilchrist *et al.*
74 *et al.* 2016). Recent data from studies with cattle also indicate that GJ-mediated cell

75 communication within the COC regulates chromatin remodeling and
76 transcriptional activity of the oocyte, and thus is of crucial importance for oocyte
77 developmental competence (Luciano *et al.* 2014). Specifically, it has been shown
78 that GJ must remain open to allow gradual chromatin remodeling *in vitro*, while
79 premature GJ interruption leads to abrupt chromatin condensation (Luciano *et al.*
80 2011; Lodde *et al.* 2013).

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

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

96 **2 MATERIALS AND METHODS**

97

98 **2.1 Chemicals**

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

103

104 **2.2 Ovaries and COC collection**

105 Ovaries of adult Nellore (*Bos indicus*), Angus (*Bos taurus*) and crossbred
106 cows were obtained at an abattoir near Sao Paulo State University – Campus of
107 Botucatu (Brazil), and ovaries from Holstein cows (*Bos taurus*) were obtained at
108 an abattoir near the University of Milan (INALCA Spa, Ospedaletto Lodigiano,
109 LO, IT 2270M CE, Italy). Ovaries transported to the laboratories in sterile saline
110 solution (0.9% NaCl) at 26-28°C. COC were aspirated from follicles of 2 to 6mm
111 in diameter with a 19-gauge needle and pooled in a 15mL conical tube. After
112 sedimentation, COC were recovered and selected with a stereomicroscope. Only
113 COC with homogeneous or slightly granulated cytoplasm and at least 5 compact
114 layers of cumulus cells were used in these experiments. COC were washed two
115 times in TCM199 with Earle's salts and 25mM HEPES, supplemented with
116 75µg/mL amikacin and 4mg/mL BSA (M199D), and groups of 20 oocytes were
117 cultured according to the experimental design (see below) in four-well dishes at
118 38.5°C and 5% CO₂ in humidified air.

119

120 **2.3 Assessment of oocyte-cumulus GJ-mediated communication**

121 Intercellular communication between the oocyte and cumulus cells was
122 assessed as previously described (Luciano *et al.* 2004). Briefly, a 3% solution of
123 Lucifer Yellow (LY) in 5mM lithium chloride was pressure injected into the oocyte,

124 and the spread of the dye into surrounding cumulus cells was monitored with an
125 inverted fluorescence microscope (Nikon Diaphot; Nikon Corp.) 10 min after the
126 injection. Obvious transfer of dye to the cumulus denoted functional GJ-mediated
127 cell communication, and the percentage of COC within each group with functional
128 cell communication was calculated.

129

130 **2.4 Assessment of germinal vesicle status**

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

139

140 **2.5 *In vitro* fertilization (IVF), embryo culture and assessment of embryo** 141 **total cell number**

142 IVF and embryo culture was performed as previously described (Lodde *et*
143 *al.* 2007). The content of a straw of cryopreserved bovine semen (only one batch
144 from the same Bull was used throughout the study) was thawed and spermatozoa
145 were separated on a 45–90% Percoll gradient. Spermatozoa were counted and
146 diluted to the final concentration of 2 x 10⁶ spermatozoa/ml in a modified Tyrode
147 solution supplemented with 0.6% BSA (fatty acid-free), 10 μ g/mL heparin, 20 μ M
148 penicillamine, 1 μ M epinephrine, and 100 μ M hypotaurine. Cumulus-oocyte

149 complexes and sperm cells were incubated for 18 hours at 38.58°C under 5%
150 CO₂ in humidified air. Presumptive zygotes were then washed, and cumulus cells
151 removed by vortexing for 1min in 500µL of a modified synthetic oviduct fluid
152 supplemented with 0.3% BSA fraction V (fatty acid-free), MEM essential and
153 nonessential amino acids, 0.72mM sodium pyruvate, and buffered with 10mM
154 HEPES and 5mM NaHCO₃. Presumptive zygotes were then transferred to the
155 embryo culture medium, which was synthetic oviduct fluid buffered with 25mM
156 NaHCO₃ and supplemented with MEM essential and nonessential amino acids,
157 0.72mM of sodium pyruvate, 2.74mM myo-inositol, 0.34mM sodium citrate, and
158 5% bovine calf serum. Incubation was performed at 38.58°C under 5% CO₂, 5%
159 O₂, and 90% N₂ in humidified air for 8 days. Blastocysts were counted and
160 morphologically classified as not expanded, expanded or hatched under a
161 stereomicroscope nine days after fertilization (Jakobsen *et al.* 2006). The
162 embryos were then fixed in 60% methanol in DPBS, stained with 1µg/mL of
163 Hoechst 33342, and cell nuclei were counted under a fluorescence microscope.

164

165 **2.6 Gene expression analysis**

166 Total RNA was extracted from cumulus cells mechanically isolated from
167 groups of 15-20 COCs subjected to the pre-IVM treatments described above
168 using the RNeasy® kit as recommended by the manufacturer. After purification,
169 RNA samples were diluted in 30 µL of RNase free water. Total RNA
170 concentrations were measured by spectrophotometry using a NanoDrop ND®
171 1000 (Thermo Scientific, Wilmington, DE, USA), and purity verified by measuring
172 the A260:A280 ratio. Total RNA (100 ng/reaction) was incubated with DNase (1
173 U/µg; Invitrogen, Sao Paulo, Brazil) and then reverse transcribed using Oligo-dT

174 primers and Omniscript (Qiagen, Mississauga, ON, CA). Samples were
175 incubated at 37°C for 60 minutes and then at 93°C for 3 min for enzyme
176 inactivation. Relative RT-qPCR analysis was performed with an ABI 7500
177 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, Sao
178 Paulo, Brazil). The final volume of the PCR mix was 24µL and 1µL of cDNA
179 sample, thermocycling conditions were: 95 °C for 10 minutes (1 cycle), denaturing
180 at 95°C for 10 seconds, followed by annealing for 1 minute (40 cycles). The
181 reference gene was *CYC-A* as previously validated in our laboratory by testing
182 several candidate genes (Machado *et al.* 2009; Caixeta *et al.* 2013). Bovine-
183 specific primers to amplify *NPR2* were designed with the PrimerQuest Tool
184 (NM_174126.2). The PCR primer sequences were as follows: *CYC-A*, 5'-
185 GCCATGGAGCGCTTTGG3' (forward) and 5'-
186 CCACAGTCAGCAATGGTGATCT3' (reverse); and *NPR2*
187 5'ATGACAGCATCAACCTGGACTGGA3' (forward) and
188 5'AGCACGAAACGACTATCCACCACA3' (reverse). Melting curve analysis
189 indicated amplification of a single amplicon. Each sample was assayed in
190 duplicate, and the relative expression values for each gene were calculated using
191 the $\Delta\Delta C_q$ method with efficiency correction and using one control sample as
192 calibrator (Pfaffl 2001). Mean efficiency values for each gene were calculated
193 from the amplification profile of individual samples with LinRegPCR software
194 (Ramakers *et al.* 2003).

195

196 **2.7 Statistical Analysis**

197 The data in the form of percentages were arcsine transformed. In order to
198 reach normal distribution, mRNA relative values were log transformed. Variance

199 homogeneity within treatments was confirmed before testing the effects of
200 treatments. The effects of treatments on the percentage of GV/GVBD oocytes,
201 percentage of COCs with functional oocyte-cumulus communication, *NPR2*
202 mRNA abundance in cumulus cells, embryo production rates and embryo cell
203 number were tested by analysis of variance (ANOVA). When ANOVA indicated
204 a significant effect of treatment, means were compared with the Tukey-Kramer
205 HSD test. These analyses were performed with the JMP software (SAS Institute,
206 Cary, NC, USA), and differences were considered significant when $P < 0.05$.

207

208 **2.8 Experimental design**

209 To measure the effects of steroids and NPPC, separately or together, on
210 GVBD rates and abundance of *NPR2* mRNA in cumulus cells, groups of 20
211 Nellore COCs were cultured for 9 hours in 400 μ L of pre-IVM medium alone
212 (Treatment 'CON'; TCM 199 containing Earle's salts, L-glutamine and NaHCO₃,
213 and supplemented with 0.4% fatty acid-free BSA, 22 μ g/mL sodium pyruvate and
214 75 μ g/mL amikacin), pre-IVM medium supplemented with 100nM NPPC
215 (Treatment 'NPPC'), pre-IVM medium supplemented with steroids and FSH
216 (Treatment 'FS'; 500ng/mL oestradiol-17 β , 50ng/mL progesterone, 50ng/mL
217 androstenedione and 10⁻⁴IU/mL FSH), or in pre-IVM medium supplemented with
218 steroids, FSH and NPPC (Treatment 'FS+NPPC'). Steroid hormones were added
219 at concentrations found in the follicular fluid of growing dominant follicles (Ireland
220 and Roche 1983; Kaneko *et al.* 1991; Beg *et al.* 2002). The concentration of
221 NPPC chosen is the lowest dose that maintained bovine oocytes in GV stage in
222 a previous study (Franciosi *et al.* 2014). An additional group of COC was included
223 in each replicate that was not cultured and served as a time 0h control for both

224 endpoints (Treatment 'CON-0h'). At the end of culture, oocytes were
225 mechanically separated from cumulus cells by repeated pipetting in PBS without
226 calcium and magnesium, and fixed and stained to assess germinal vesicle status
227 as described above. Cumulus cells were transferred to 1.5mL tubes, collected by
228 centrifugation for 5 min at 700g and frozen at -80°C in 350µL of RNA extraction
229 lysis buffer (RNeasy® kit; Qiagen, Mississauga, ON, Canada) to assess the
230 effects of treatments on the abundance of *NPR2* mRNA. These experiments were
231 performed four times.

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

242 Finally, to assess the effects of steroids and NPPC in a pre-IVM culture
243 step on *in vitro* embryo production, groups of 20 oocytes from Nellore, Angus or
244 crossbred cows were cultured in CON, NPPC, FS and FS+NPPC media as
245 described above for 9 hours, and then subjected to IVM in 400µL IVM medium
246 containing 10ng/mL insulin-like growth factor 1 (IGF-1), 100ng/mL AREG, 10⁻²
247 IU/mL FSH and concentrations of oestradiol-17β (50ng/mL) and progesterone
248 (150ng/mL) observed in bovine follicles after the LH surge (Fortune and Hansel

249 1985; Komar *et al.* 2001). After 24h, oocytes underwent *in vitro* fertilization (IVF)
250 as described above. This experiment was replicated four times and in each
251 replicate COCs of a single breed were used; Nellore and crossbred COCs were
252 used in one replicate each, and Angus COCs were used in two replicates. The
253 numbers of COCs subjected to each treatment were 195, 196, 197 and 195 for
254 CON, NPPC, FS and FS+NPPC, respectively.

255

256 **3 RESULTS**

257

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

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

268 Culture of *Bos taurus* COC in pre-IVM medium significantly reduced the
269 proportion of oocytes in GV arrest compared with non-cultured controls (Fig 2a),
270 and supplementation with NPPC but not steroids+FSH significantly increased the
271 proportion of GV-arrested oocytes. The combination of NPPC and steroids+FSH
272 was not different from NPPC alone. Similarly, culture in pre-IVM medium
273 significantly reduced the percentage of COCs with functional oocyte-cumulus

274 cells GJ-mediated communication (Fig. 2b), and supplementation with NPPC but
275 not FSH+steroids significantly increased the percentage of COCs with open
276 oocyte-cumulus cells GJ-mediated communication. The combination of NPPC
277 with steroids+FSH was not different from NPPC alone. However, the combination
278 of NPPC with steroids+FSH, but not NPPC alone, promoted GV arrest and
279 oocyte-cumulus cells GJ-mediated communication at levels not different from
280 non-cultured controls (Fig.2ab).

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

291

292 **4 DISCUSSION**

293

294 Previous studies have shown that NPPC inhibits oocyte nuclear
295 maturation in several species including the mouse, cow, pig and cat (Zhang *et al.*
296 2010; Franciosi *et al.* 2014; Blaha *et al.* 2015; Zhong *et al.* 2015). Studies in mice
297 suggest that oestradiol-17 β increases the sensitivity of cumulus cells to NPPC
298 and is required for NPPC to inhibit meiotic resumption (Zhang *et al.* 2011). There

299 are no previous studies exploring the potential interaction of NPPC and a
300 combination of steroid hormones at physiological levels in the regulation of oocyte
301 nuclear maturation. Here we show novel evidence that the presence of oestradiol-
302 17 β , progesterone and androstenedione enhances the ability of NPPC to delay
303 germinal vesicle breakdown and to maintain oocyte-cumulus cells GJ-mediated
304 communication in the bovine COC by mechanisms involving an increase in the
305 sensitivity of cumulus cells to NPPC. In addition, we demonstrate that
306 supplementation of the culture medium with physiological concentrations of
307 steroids in a pre-IVM culture step improves embryo quality after IVF.

308 Pre-IVM cultures with phosphodiesterase inhibitors or NPPC can prolong
309 meiotic arrest and improve embryonic developmental competence in cattle (Albuz
310 *et al.* 2010; Franciosi *et al.* 2014). A previous study in mice demonstrated that
311 both oestradiol-17 β and testosterone individually inhibit germinal vesicle
312 breakdown, while increasing the expression of *NPR2* in cumulus cells. In the
313 same study, oestradiol-17 β was required to sustain the ability of NPPC to
314 stimulate cGMP production during culture (Zhang *et al.* 2011). Differently than in
315 mice, oestradiol-17 β did not synergize with NPPC to delay GVBD in cattle
316 (Franciosi *et al.* 2014). Nevertheless, data from the present study suggest that
317 the presence of all three major steroid hormones at approximately intrafollicular
318 concentrations enhances the ability of NPPC to delay GVBD and to prolong
319 oocyte-cumulus cells GJ-mediated communication in the cow. This suggests a
320 physiological role for intrafollicular steroids in the regulation of oocyte nuclear
321 maturation through NPPC signaling in cattle, although oestradiol-17 β alone may
322 not be as effective as in mice to sensitize bovine cumulus cells to NPPC (Zhang
323 *et al.* 2011; Franciosi *et al.* 2014).

324 Interestingly, in the present study the combination of steroids+FSH and
325 NPPC induced a two-fold increase in *NPR2* mRNA abundance in cumulus cells
326 compared with treatment with NPPC alone. Although we have not measured
327 *NPR2* protein or activity, we speculate that the synergistic effect of steroids and
328 NPPC in cattle is at least in part a consequence of increased sensitivity of
329 cumulus cells to NPPC. This is consistent with previous data in mice, in which
330 induction of greater *NPR2* mRNA abundance was associated with higher *NPR2*
331 protein expression and cGMP production by cumulus cells (Zhang *et al.* 2011).

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

342 In this study, we aimed for a more physiological system capable of
343 sustaining meiotic arrest and oocyte-cumulus cells GJ-mediated communication,
344 therefore steroid hormones were added to the pre-IVM medium at concentrations
345 observed in the follicular fluid of growing dominant follicles (Ireland and Roche
346 1983; Kaneko *et al.* 1991; Beg *et al.* 2002). Our data further support that the
347 administration of low concentrations of FSH appear to be effective in promoting
348 intercellular communication within the cumulus-oocyte complex probably by

349 regulating Cx43 phosphorylation in a way that favors GJ coupling (Atef *et al.*
350 2005; Luciano *et al.* 2011; El-Hayek and Clarke 2015).

351 During final antral follicle growth, oestradiol-17 β levels are high and
352 progesterone levels are low, but around the time of ovulation the LH surge
353 triggers the differentiation of theca and granulosa cells to small and large luteal
354 cells, and steroidogenesis is directed towards progesterone production (Fortune
355 and Hansel 1985; Komar *et al.* 2001). Higher concentrations of progesterone and
356 lower concentrations of oestradiol-17 β in the preovulatory follicle were associated
357 with better cumulus expansion and blastocyst production (Aardema *et al.* 2013).
358 Moreover, progesterone signaling during IVM is necessary for cumulus
359 expansion and oocyte developmental competence (Aparicio *et al.* 2011). These
360 observations led us to increase progesterone concentration and to decrease
361 oestradiol-17 β concentration in the IVM medium. Also aiming for a more
362 physiological induction of oocyte maturation, IVM was performed in the presence
363 of AREG and FSH at concentrations lower than the supraphysiological levels
364 commonly used (Richani *et al.* 2014; Sugimura *et al.* 2014). Supplementation of
365 the pre-IVM culture medium with NPPC and steroids, separately or combined,
366 did not affect blastocyst rates in the present study. On the other hand,
367 supplementation of the pre-IVM medium with steroids+FSH or steroids+FSH
368 combined with NPPC increased blastocyst total cell number, the greatest
369 numerical increase being observed with the combination. Since supplementation
370 of the pre-IVM medium with steroids+FSH did not significantly affect GVBD or
371 oocyte-cumulus cells GJ-mediated communication in the present study, the
372 beneficial effect of steroids on developmental competence appear to involve
373 other processes in cumulus cells. As mentioned above, progesterone signaling

374 stimulates cumulus expansion in cattle and is crucial for developmental
375 competence (Aparicio *et al.* 2011). In mice, oestrogens increase transcription of
376 a variety of genes regulating expansion, metabolism, proliferation and EGF-
377 stimulated pathways in cumulus cells (Sugiura *et al.* 2010; Emori *et al.* 2013). We
378 are not aware of any studies assessing the effects of different steroids combined
379 on cumulus cell function.

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

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

399

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408

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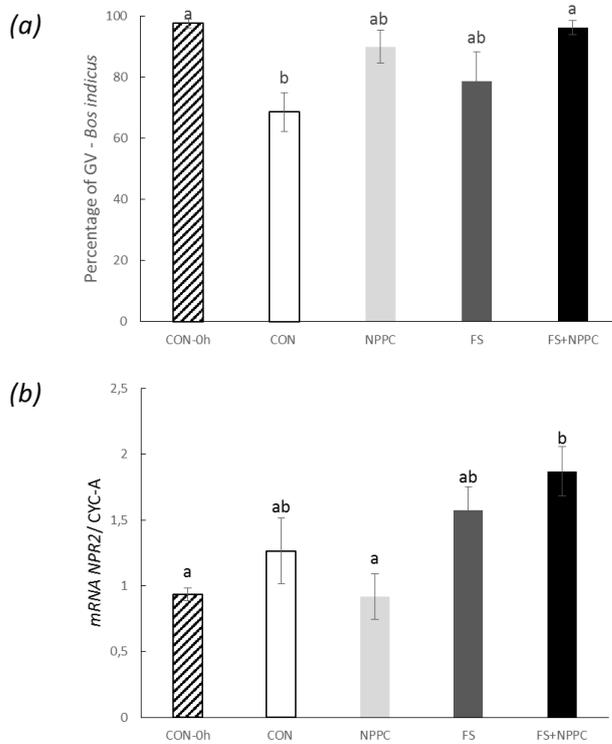
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570 FIGURES

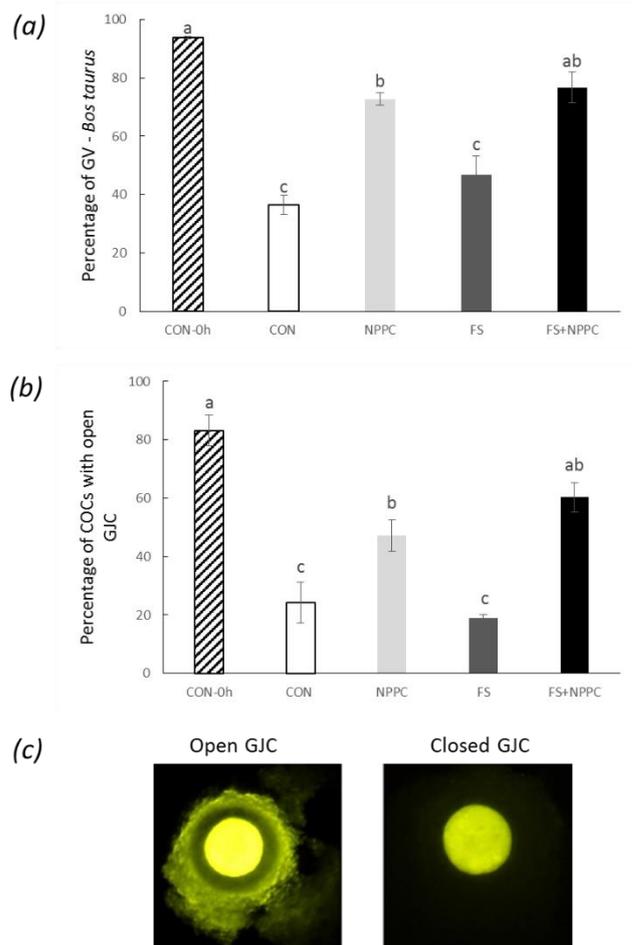
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573 Figure 1. Effects of steroid hormones and NPPC on (a) the percentage of GV-
574 arrested oocytes and (b) *NPR2* mRNA abundance in cumulus cells after culture
575 for 9 hours. *Bos indicus* COCs were cultured with treatments CON (base medium
576 only), NPPC (base medium + NPPC), FS (base medium + steroids/FSH) and
577 FS+NPPC (base medium + steroids/FSH + NPPC). Non-cultured COCs were
578 included in the analyses as a time 0h control (CON-0h). Experiments were
579 performed on 4 independent replicates with 15-20 oocytes per treatment).
580 Different letters indicate statistically significant differences (P<0.05).

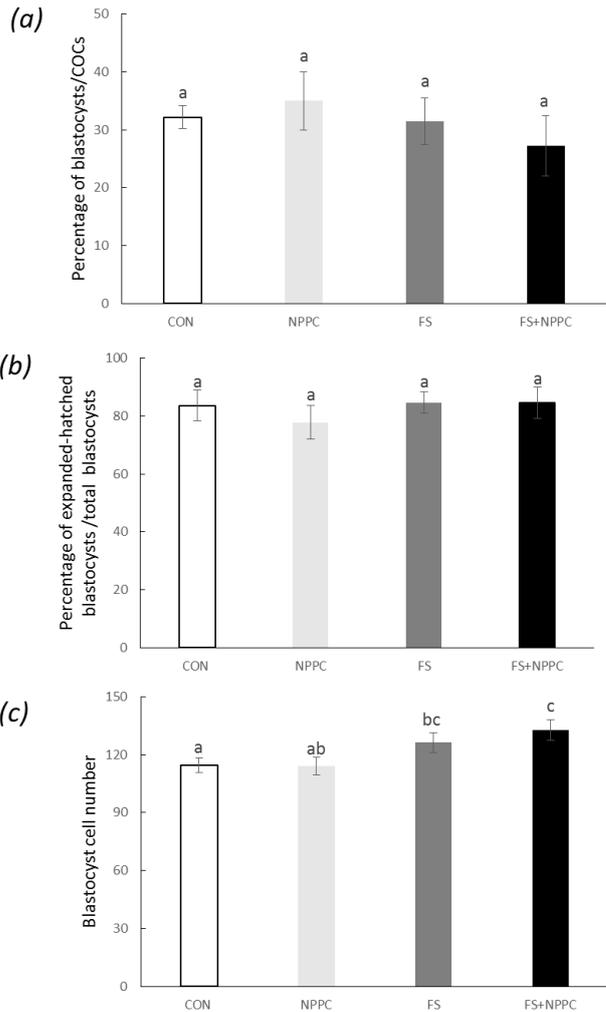
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583 Figure 2. Effects of steroid hormones and NPPC on the percentage of GV-
 584 arrested oocytes **(a)**, and percentage of COCs with functional oocyte-cumulus
 585 communication **(b)** after culture for 9 hours. *Bos taurus* COC were cultured with
 586 treatments CON (base medium only), NPPC (base medium + NPPC), FS (base
 587 medium + steroids/FSH) and FS+NPPC (base medium + steroids/FSH + NPPC).
 588 Non-cultured COCs were included in the analyses as a time 0h control (CON-
 589 0h). Experiments were performed in 3 independent replicates with 15-20 oocytes
 590 per treatment. Different letters indicate statistically significant differences
 591 ($P < 0.05$). **(c)** Representative images showing COCs with open or closed GJ
 592 oocyte-cumulus communication as detected by transfer of Lucifer Yellow
 593 between the oocyte and cumulus cells.

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595

596 Figure 3. Effects of steroid hormones and NPPC added separately or together in
 597 a pre-IVM culture on embryo production as measured by the percentage of
 598 blastocysts in relation to total oocytes **(a)**, percentage of expanded and hatched
 599 blastocysts in relation to total blastocysts **(b)** and blastocyst cell number **(c)**.
 600 Bovine COCs were cultured for 9 hours with pre-IVM treatments CON (base
 601 medium), NPPC (base medium+NPPC), FS (base medium+steroids/FSH) and
 602 FS+NPPC (base medium+steroids/FSH+NPPC), followed by IVM, IVF and
 603 embryo culture for 8 days. This experiment was replicated 4 times with 195, 196,
 604 197 and 195 COCs allocated to treatments CON, NPPC, FS and FS+NPPC,
 605 respectively. Different letters indicate statistically significant differences (P
 606 <0.05).