Expanded equine cumulus-oocyte complexes exhibit higher meiotic competence and lower glucose consumption than compact cumulus-oocyte complexes

Running head: Equine oocyte glucose metabolomics.

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Abstract

Equine cumulus-oocyte complexes (COCs) are classified as compact (cCOC) or expanded (eCOC) and vary in their meiotic competence. This divergence could be related to different glucose metabolism. To test this hypothesis eCOCs, cCOCs, and expanded or compact mural granulosa cells (EC and CC respectively) were matured *in vitro* for 30 hours and the maturation rate, glucose metabolism, and expression of genes involved in glucose transport, glycolysis, apoptosis and meiotic competence were determined. Significant differences were found between eCOCs and cCOCs maturation rates (50% vs. 21.7 %; n = 192 and 46 respectively, $p < 0.001$), glucose consumption (1.8 ± 0.5 vs. 27.9 ± 5.9 nmol/COC; mean ± SEM), pyruvate production (0.1 ± 0.0 vs. 2.4 ± 0.8 nmol/COC; mean ± SEM) and lactate production (4.7 ± 1.3 vs. 64.1 ± 20.6 nmol/COC; mean ± SEM) respectively ($p < 0.05$). Moreover, similar glucose consumption was observed for EC and CC. Hyaluronan binding protein (*TNFAIP6)* expression was increased in eCOCs and EC, solute carrier family 2 (facilitated glucose transporter) member 1 *(SLC2A1)* was increased in eCOCs, while glycolysis-related enzymes and solute carrier family 2 (facilitated glucose transporter) member 3 (*SLC2A3*) expression did not vary between COCs or mural granulosa cell type. Our data demonstrate that metabolic and genomic differences exist between eCOCs and cCOCs and mural granulosa cells in the horse.

Additional Keywords: *in vitro* maturation, glycolysis, nuclear magnetic resonance, horse.

Introduction

Oocyte *in vitro* maturation (IVM) was achieved for the first time in rabbits by Pincus and Enzmann (1935). Mammalian oocytes can be harvested directly from excised ovaries or removed from the ovaries of live females by transvaginal aspiration (Hinrichs 2010b; Hourvitz *et al.* 2015) and have to reach the metaphase II stage (MII) prior fertilization (Downs 2015). The process of oocyte maturation involves the resumption of meiosis, the expansion of the cumulus cells and the maturation of the cytoplasm (Sutton-McDowall *et al.* 2010). Oocytes are enclosed by numerous layers of granulosa cells which differentiate into two populations spatially and functionally: cumulus cells that surround the oocyte and mural granulosa cells that line the follicular wall; in addition, both

granulosa cell lines differ in their metabolism and expression of glycolysis-related genes, establishing a complex interplay with the oocyte (Sugiura *et al.* 2005).

A core feature defining the capacity of the individual oocyte to reach MII, also known as meiotic competence, is the initial oocyte quality (Keefe *et al.* 2015). Oocyte quality is generally judged by the appearance of the oocytes under the microscope. Equine oocytes are classified as compact or expanded and are distinguished by the appearance of their cumulus and the degree of expansion of the mural cells present around the COCs (Hinrichs 2010b; Gonzalez-Fernandez et al. 2015) and their meiotic competence differ vividly; while 71% of expanded oocytes mature in culture, only 21% of compact oocytes (approx.) reach the MII stage (Hinrichs 2010a).

Disregarding the species in study, these parameters are subjective. Even when only good quality oocytes are used, the number of mature oocytes and embryos/births obtained will not reach 100% of the initial oocyte pool (Gandolfi and Brevini 2010). In this regard, a lot of effort is being put into identifying factors that define the oocyte meiotic competence including expression and transcription of candidate genes (Mohammadi-Sangcheshmeh *et al.* 2014; Scarlet *et al.* 2016), follicular fluid analysis (Gerard *et al.* 2002; Gérard *et al.* 2014) or oocyte metabolomics (Sessions-Bresnahan *et al.* 2016) among others.

Metabolomics allows for the non-invasive study of factors present in the medium that are secreted or consumed as a result of the cumulus-oocyte complex (COC) metabolism (Nel-Themaat and Nagy 2011). Carbohydrate metabolism, namely glucose and pyruvate, deeply influence the meiotic competence of the oocyte in many mammalian species (Downs and Hudson 2000; Herrick *et al.* 2006; Johnson *et al.* 2007; Songsasen *et al.* 2007). In addition, regulation of genes involved in glucose metabolism such as hexokinase (*HK*), phosphofructokinase (*PFK*), pyruvate dehydrogenase (*PDH*) and lactate dehydrogenase (*LDH*) are important for assessing glucose metabolism in any living cell including the oocyte (Kumar *et al.* 2013) and perturbations affecting oocyte's glucose uptake and metabolism impedes meiosis progression (Zheng *et al.* 2007).

Herein, we aimed to study the uptake and metabolism of glucose as well as its possible association with the meiotic competence of expanded and compact equine oocytes. To this end, equine expanded (eCOCs) and compact cumulus oocyte-complexes (cCOCs) and mural granulosa cells were cultured separately. After IVM, glucose metabolism was studied by: 1) assessment of glucose consumption and derived metabolites present in the

culture medium using nuclear magnetic resonance (NMR) and 2) expression of candidate genes involved in glucose transport, glycolysis, apoptosis and meiotic competence by quantitative polymerase chain reaction (qPCR). In addition, meiosis completion of eCOCs and cCOCs was also assessed by fluorescence microscopy.

Materials and methods

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich Inc. (Barcelona, Spain) unless otherwise stated.

Oocyte harvesting and in vitro maturation

The base medium used for oocyte *in vitro* maturation was Tissue Culture Medium 199 (TCM-199)added with 25 mM HEPES (M2520; Sigma, Barcelona, Spain), 25 mM Bicarbonate and 25 μg/ml gentamicin. Bicarbonate was not added when oocytes were handled in the laminar flow hood and the osmolarity was adjusted by NaCl addition. The pH was adjusted to 7.4 and osmolarity was set to 280 mOsm/Kg (approx).

Equine ovaries were obtained from a slaughterhouse. The ovaries were placed in a thermic box and transported to the laboratory at room temperature (RT; 20-22°C; 1 hour of transport approx.). Oocytes were obtained by follicular scraping as previously reported (Gonzalez-Fernandez *et al.* 2015). Briefly, each visible follicle was cut with a scalpel blade, meticulously scraped using a bone curette and rinsed on a 35 mm Petri dish with PBS containing 1 U/ml heparin, 25 μg/ml gentamicin and 0.1% polivinilalcohol (PVA). Each follicle was scraped in a separate dish to allow for proper COC and granulosa cell classification. COCs were searched under a dissection microscope, and classified either as cCOC or eCOC based on the morphology of the cumulus and mural granulosa cells observed in the dish; if any signs of expansion were observed, the COC was considered as eCOC while cCOC showed no signs of expansion (Suppl. 1). Twenty microliters of medium containing non-disaggregated mural granulosa cells were aspirated from each of the dishes corresponding to expanded or compact follicles; if

expanded and compact cells coexisted in a dish, special care was used to retrieve both. Expanded mural granulosa cells (EC) and compact (CC), cCOCs and eCOCs were then separately transferred to a Petri dish containing TCM-199 added with 10% fetal bovine serum (FBS) until the end of the process. From each dish containing EC and CC two hundred microliters of the pooled granulosa cells were aspirated. COCs and mural granulosa cells were separately held overnight at room temperature (≈22°C) for a maximum of 16 hours in TCM-199 supplemented with 25 mM bicarbonate and 20% FBS (holding medium or HM) as previously described (Hinrichs 2010b; Martino et al. 2014; Gonzalez-Fernandez et al. 2015). A maximum of 25 oocytes were placed in a 4-well Nunc® plate containing 500 µl HM, filled to the border with mineral oil, sealed using parafilm and wrapped in aluminum foil. The following morning, COCs and mural granulosa cells were transferred to 4-well Nunc® plates containing 250 µl of maturation medium covered with mineral oil (TCM supplemented with 25 mM bicarbonate, 5 mU⁄ml of Follicle stimulating hormone (FSH; Ref. F8174) from sheep pituitary and 10% FBS). The samples were then incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 hours. For all the experiments, the maturation medium was allowed to equilibrate for at least 3 hours prior the beginning of the experiment. After oocyte maturation, 200 µl of media of each COC and mural granulosa cell groups were retrieved and stored at -80°C for later nuclear magnetic resonance analysis. All the granulosa cells from EC and CC groups were retrieved and stored at -80 ºC for protein quantification. A total of 99 eCOCs (15-25 oocytes per well) and 17 cCOCs (1-4 oocytes per well) were placed in culture and the supernatants were retrieved for NMR experiments. Sixteen replicates of the IVM experiment were performed for DNA configuration assessment including the oocytes used for NMR; for each replicate, ovaries from 2-5 different mares were used and a total of 192 eCOCs (15-25 oocytes per well) and 46 cCOCs (1-8 oocytes per well) were evaluated.

Oocyte degeneration and maturational status evaluation

Equine oocytes were evaluated after maturation *in vitro*. First, cumulus cells were removed from the COC with PBS supplemented with 0.2% PVA (PBS+PVA) by meticulous pipetting using decreasing diameter bore glass pipettes (Vitrolife, Götteborg, Sweden) until no granulosa cells were visualized on the zona pellucida. Denuded oocytes were then fixed in 4% formaldehyde in PBS+PVA for 12 hours at 4°C. Then, the oocytes were thoroughly

washed in PBS+PVA and stained with 5 µg/ml of Hoechst 33342 at 37°C for 10 minutes in the dark. The oocytes were mounted on slides using glycerol and a coverslip, sealed with nail polish and allowed to air dry. DNA integrity and conformation were classified as follows: germinal vesicle, metaphase I or metaphase II using an Olympus BX41 fluorescence microscope (New Hyde Park, NY, USA using a 40× objective based on previously validated criteria (Hinrichs 2010a)). Oocytes were considered as degenerated when no DNA was present or if unidentifiable chromatin configurations were found.

RNA Extraction, reverse transcription and quantification of mRNA transcript abundance

Gene expression was analyzed in a) matured eCOCs (n = 30), b) matured cCOCs (n = 21), a representative sample of c) compact mural granulosa cells and d) expanded mural granulosa cells subjected to IVM conditions, retrieved on 4 different days. COCs and granulosa cells were pooled and 7-10 COCs per replicate were used (3 replicates for each COC and mural granulosa cell type).

Expanded and compact COCs and mural granulosa cells were processed separately. After IVM, samples were thoroughly washed in PBS+PVA, then placed in 20 µl of RNAlater (Ambion®, Thermo Fisher Scientific Inc., Oslo, Norway), allowed to equilibrate for 5 min at RT, plunged in liquid nitrogen and stored at -80ºC until RNA extraction (Romar *et al.* 2011). Before RNA extraction, samples were thawed and centrifuged at 300 g for 5 min to eliminate the RNA later. Poly (A) RNA was extracted using the Dynabeads ® mRNA DIRECT™ Micro Kit (Ambion®, Thermo Fisher Scientific Inc., Oslo, Norway) as previously reported (Bermejo-Alvarez et al. 2008). In brief, after samples were incubated in lysis buffer for 10 min with Dynabeads, poly (A) RNA attached to the Dynabeads was magnetically extracted and washed twice with washing buffer A and washing buffer B. RNA was obtained after elution with Tris-HCl. The RT reaction was then performed following the manufacturer's instructions (Epicentre Technologies Corp., Madison,Wis., U.S.A.); to prime the RT reaction and to produce cDNA poly (T) primers, random primers, and MMLV High Performance Reverse Transcriptase enzyme in a total volume of 40 µl were mixed. The tubes containing the mix were heated to 70ºC for 5 min to denature the secondary RNA structure and after the addition of 50 units of reverse transcriptase, the RT mix was completed. Retrotranscription was performed by incubating at 25ºC for 10 min to favor the annealing of random primers,

154 followed by 37ºC for 60 min to allow the RT of RNA, and finally 85ºC for 5 min to denature the enzyme. After
155 cDNA synthesis, the samples were diluted to 55 µl in 10 mM Tris –HCl (pH 7.5).

156 Three cDNA samples were used per experimental group and all qPCR reactions were carried out in duplicate in
157 the Rotorgene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia). The experiment was conducted
158 to contrast the relative levels of each transcript and the housekeeping gene, histone H2A family, member Z
159 (*H2AFZ*), in each sample. PCR was carried out by adding a 2 µl aliquot of each cDNA sample to the PCR mix
160 (GoTaq qPCR Master Mix, Promega Corporation, Madison,Wis., USA) containing specific primers selected to
161 amplify the selected genes: Tumor necrosis factor alpha-induced protein 6 (TNFAIP6); Growth differentiation
162 factor 9 (*GDF9*); Bone Morphogenetic Protein 15 (*BMP15*); Tumor necrosis factor receptor superfamily, member
163 6 (*FAS*); Fas Ligand (*FASLG*); BCL2-associated X protein (*BAX*), transcript variant X2, mRNA; B-Cell
164 CLL/Lymphoma 2 (*BCL2L1*); solute carrier family 2 (facilitated glucose transporter) member 1 (*SCL2A1*, former
165 *GLUT1*); member 3 (*SCL2A3* former *GLUT3*); Phosphofructokinase, platelet (*PFKP*); Pyruvate dehydrogenase
166 kinase, isozyme 3 (*PDK3*); Lactate deshydrogenase A (*LDHA*); 18 S ribosomal RNA (*RN18S*); Glyceraldehyde-3-
167 phosphate dehydrogenase (*GAPDH*) and H2A histone family, member Z (H2AFZ).

168 Primers were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primersblast/) to span exon-
169 exon boundaries when possible. Primer sequences and the approximate sizes of the amplicons are shown in Table
170 1. Cycling conditions were as follows: 94 ºC for 3 min followed by 35 cycles of 94ºC (15 sec), 56ºC (30 sec),
171 72ºC (10 sec) and 10 sec for fluorescence acquisition. Each pair of primers was tested to achieve efficiencies
172 close to 1; to quantify their expression levels the comparative cycle threshold C(q) method was used (Schmittgen
173 and Livak 2008). Fluorescence was acquired in each cycle at a temperature higher than the melting temperature of
174 primer dimers to avoid primer artifacts (specific temperatures for each product varied from 80–86 °C). For each
175 sample the threshold cycle or the cycle during the logarithmic linear phase of the reaction at which fluorescence
176 increased above background was determined. The ΔCT value was determined as follows: the endogenous control
177 (H2AFZ) C(q) value was subtracted for each sample from each gene C(q) value of the sample. Determination of
178 ΔΔCT involved using the highest sample ΔC(q) value as a constant to subtract from all other ΔC(q) sample values.
179 The equation 2–ΔΔCQ was used to determine the fold-changes in the relative gene expression of the target.

Nuclear Magnetic Resonance

After thawing, the supernatants (200 µl) of the COCs and mural granulosa cells collected after IVM were subjected to proton nuclear magnetic resonance (1H-NMR); the 1H-NMR was run at 14.1 T, 25ºC using a 600 MHz Bruker Avance spectrometer (Bruker Biospin, Germany), to determine glucose consumption, lactate and pyruvate production as routinely used by our team (Martins *et al.* 2015). Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ, ppm); lactate (doublet, 1.33); pyruvate (singlet, 2.36); H1-α glucose (doublet, 5.22). Spectra analysis was performed offline. The relative areas of 1H-NMR resonances were quantified using the curve-fitting routine using the NutsProTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA). Results were expressed as metabolite consumption or production per µg of total protein (pmol/µg protein) in EC and CC and as nmol/COC for eCOCs and cCOCs. The NMR spectra for eCOCs and cCOCs were normalized depending upon the number of COCs placed per well and maturation day; 99 eCOCs (15-25 oocytes per well) and 17 cCOCs (1-4 oocytes per well) were placed in culture and their metabolic by-products were analyzed. A total of 7 groups retrieved on 3different days were analyzed (7 replicates in total).

Total protein quantification

Total proteins of compact mural granulosa cells (CC) and expanded mural granulosa cells (EC) were isolated using RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS with freshly added 1 mM phenylmethylsulfonyl fluoride, supplemented with 1% protease inhibitor cocktail, 30 µl/ml aprotinin and 100 mM sodium orthovanadate). The lysed mural granulosa cells were allowed to stand for 15 minutes on ice, and the suspension was centrifuged at 14000g for 20 minutes at 4ºC. The resulting pellet was discarded and protein concentration was determined using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) following the

instructions provided by the supplier. The protein concentration obtained was used to normalize the metabolite consumption and production of mural granulosa cells analyzed by NMR.

Statistical analysis

The proportions of oocytes showing different chromatin configurations were compared among groups by Chi-square test with the Yates correction for continuity. The Fisher's Exact Test was used when a value of less than 5 was expected in any treatment. The differences in fold increase expression of the genes under study followed a normal distribution and had homogeneous variances, thus, a student t-test was used to compare pairs of values. When the normality test failed a Mann-Whitney Rank Sum Test was conducted using Sigma Plot software version 11.0. Data obtained by NMR and qPCR were analyzed using the Sigma Stat software package (Jandel Scientific, San Rafael, CA). A Student t-test was performed to study the differences in metabolites between cCOCs and eCOCs and compact and expanded mural granulosa cells; $p < 0.05$ was considered as significant.

Results

Equine oocyte maturation and degeneration rates

Oocyte maturation was evaluated after 30 h in maturation medium. All IVM experiments run in our laboratory from June 2014 to April of 2015 were included (16 IVM experiments in total; 46 cCOCs and 192 eCOCs). Significant differences were found between eCOCs and cCOCs regarding maturation rates (50% vs. 21.7% for eCOCs and cCOCs respectively; $p < 0.001$) and degeneration rates (33.9% vs. 60.9% for eCOCs and cCOCs respectively; $p < 0.001$) after IVM. No significant differences were found between eCOCs and cCOCs for germinal vesicle (GV) or metaphase I (MI) stages ($p > 0.05$; Table 2).

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230 *Characterization of glucose metabolism in cCOCs and eCOCs, and compact or expanded mural granulosa cells*

231

232 Glucose consumption vividly varied between cCOCs and eCOCS and between compact (CC) and expanded (EC)
233 mural granulosa cells. cCOCs and CC consumed significantly higher amounts of glucose compared to expanded
234 samples (27.9 ± 5.9 vs. 1.8 ± 0.5 nmol/COC, respectively; mean ± SEM) and EC (40.0 ± 12.0 vs. 0.7 ± 0.0
235 pmol/µg protein, respectively; mean ± SEM) (Fig. 1A). Likewise, cCOCs showed a higher metabolic rate than
236 eCOCs, as pyruvate (2.4 ± 0.8 vs. 0.1 ± 0.0 nmol/COC, cCOCs vs. eCOCs; mean ± SEM) and lactate production
237 (64.1 ± 20.6 vs. 4.7 ± 1.3 nmol/COC, cCOC vs. eCOC respectively; mean ± SEM) were greater in cCOCs (Fig.
238 1B and 1C; $p < 0.05$). The same pattern was observed for CC and EC, as pyruvate (5.1 ± 2.7 vs. 0.1 ± 0.0 pmol/µg
239 protein, CC vs. EC respectively; mean ± SEM) and lactate production (42.1 ± 23.7 vs. 2.0 ± 5.0 pmol/µg protein,
240 CC vs. EC respectively; mean ± SEM) were enhanced in CC (Fig. 1B and 1C; $p < 0.05$).

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242 *Relative mRNA expression of glycolysis, meiotic competence and apoptosis related genes*

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244 In view of the previously obtained data, the quantitative expression of candidate genes related to apoptosis,
245 glucose metabolism and meiotic competence were compared between eCOCs and cCOCs and between EC and
246 CC. We first studied the gene expression levels of three housekeeping genes (*H2AFZ*, *RN18S*, and *GAPDH*) to
247 identify the housekeeping with minimal variability under our experimental conditions (compact or expanded
248 COCs, and compact or expanded mural granulosa cells). In our experimental setting H2AFZ was the most suitable
249 housekeeping gene for normalizing mRNA levels (data not shown). The expression of this housekeeping gene was
250 found to be directly proportional to the amount of mRNA present in non-normalized reverse transcription
251 reactions; hence, H2AFZ was selected for the quantification of the mRNA transcripts. In relation to mRNA
252 expression in COCs, although maturation and degeneration rates were significantly different between cCOCs and

eCOCs, only the expression of *TNFAIP6* varied between eCOCs and cCOCs as shown in Fig. 2 (3.17 ± 0.47 vs. 1.0 ± 0.60 fold increase; mean ± SEM; respectively, $p < 0.05$).

When the overall expression of the oocyte competence and glucose metabolism related genes were compared between CC and EC only the hyaluronan binding protein (TNFAIP6) and the glucose transporter 1 (*SLC2A1)* exhibited differences and were consistently enhanced in EC; for *TNFAIP6* (1.0 ± 0.76 vs. 2.13 ± 0.80 fold increase; mean ± SEM respectively, $p < 0.05$) and for *SLC2A1* (1.0 ± 0.69 vs. 16.33 ± 0.79 fold increase; mean ± SEM respectively, $p < 0.05$). Regarding the apoptosis-related genes, a higher expression was observed in CC compared to EC for *FAS* (4.90 ± 0.92 vs. 1.0 ± 0.36 fold increase; mean ± SEM respectively, $p < 0.05$) and *BAX*/*BCL2L1* (2.50 ± 1.25 vs. 0.60 ± 0.20 fold increase; mean ± SEM respectively, $p < 0.05$) (Fig. 3).

Discussion

During the first part of our study, the meiotic competence of eCOCs and cCOCs was assessed and a higher degeneration rate was observed in cCOCs compared to eCOCs after IVM (60.9% vs. 33.9% respectively; $p < 0.05$). Previous studies have demonstrated that compact oocytes are retrieved from juvenile follicles and usually degenerate during IVM (65% approx.), while expanded oocytes come from atretic follicles, experience lower degeneration and exhibit higher meiotic competence after IVM (Hinrichs 2010a). Also, coinciding with previous reports, in our study cCOCs showed lower meiotic competence than eCOCs (Hinrichs 2010b; Hinrichs 2010a). In view of these data, our maturation and degeneration rates for equine eCOCs and cCOCs are in agreement with the literature and thus, our maturation system is fully validated. In addition, the percentage of eCOCs and cCOCs retrieved in the present study for the NMR and IVM experiments also demonstrate that cCOCs are accurately separated from eCOCs. Although in general cCOCs represent 30% approx. of the COCs retrieved and eCOCs account for 60%, the yield varies between laboratories as the criteria are subjective (Hinrichs 2010a; Gonzalez-Fernandez et al. 2015). However, a retrospective study of all the equine follicles scraped from January of 2014 to December of 2015 in our laboratory (1312 oocytes in total) resulted in 326 cCOCs (24%) and 986 expanded (76%) excluding the degenerated oocytes that were discarded and not accounted for (data not shown). These data

reflect that, in our setting, the classification criteria are well established and repeatable among oocyte harvesting sessions.

Previous studies have demonstrated that the oocytes' glycolytic activity is intimately related to its capacity to resume meiosis (Sutton *et al.* 2003; Sutton-McDowall *et al.* 2010). As denuded oocytes have very poor capacity to metabolize glucose, the granulosa cells surrounding the oocyte are in charge of glucose metabolism (Sutton-McDowall *et al.* 2010) and pyruvate and lactate production during IVM (Donahue and Stern 1968; Billig *et al.* 1983). Our data demonstrate that in equine cCOCs and compact mural granulosa cells, glucose consumption and production of pyruvate and lactate are significantly enhanced compared to their expanded counterparts (Fig. 1A, 1B and 1C; $p < 0.05$). Interestingly, in contrast with our findings, all the previously mentioned carbohydrates are known to be involved in meiosis resumption of COCs in various species (Downs *et al.* 2002; Downs 2015). For example, glucose plays a key role in nuclear and cytoplasmic maturation of murine COCs (Donahue and Stern 1968; Xie *et al.* 2016), pyruvate addition to the IVM medium increases meiosis resumption of bovine and murine COCs (Downs and Hudson 2000; Geshi *et al.* 2000) and lactate is actively produced during IVM of murine COCs (Downs *et al.* 1996). Coinciding with our findings, a brief report has demonstrated that equine cCOCs consume significantly higher amounts of glucose compared to eCOCs but produce lactate at similar rates (Len *et al.* 2016); however, in our study, the compact specimens consistently produced higher lactate quantities (Fig. 1C). This divergence could be due to the different analytical method used (NMR vs. a chemical analyser), to the lower number of COCs used in their study or to the marked differences that exist between laboratories in the equine oocyte classification standards. In addition, in the study by Len *et al.* (2016) insulin-transferrin selenium and significantly higher concentrations of FSH were used (5 mU/ml in the present study vs. 1000 U/ml according to Puregon® manufacturer). It has been demonstrated that both components influence oocytes' glucose metabolism (Janicot and Lane 1989; Downs and Mastropolo 1994), and could also help to explain the apparent divergences found. A limitation of the present study is the lower number of cCOCs used compared to eCOCs but the sensitivity of the technique used (NMR) is high, and has been validated in similar settings (Singh and Sinclair 2007). In addition, other reports have also demonstrated different glycolytic capacity of equine COCs depending upon DNA content disregarding their maturational status or the degree of cumulus expansion (Lewis *et al.* 2015). However, clear differences in glucose consumption of eCOCs and cCOCs and mural granulosa cells are reported

306 in the present study. Our data show that compact specimens have higher metabolic activity when compared to
307 eCOCs and expanded mural granulosa cells, but this increased metabolism does not correlate with higher meiotic
308 competence in equine oocytes (Fig. 1 and Table 2). This seems to be species-specific as while, in general, factors
309 such as increased glucose consumption and cumulus compaction are related to improved developmental outcome
310 in human oocytes (Sutton et al. 2003; Sutton-McDowall et al. 2010), this does not occur in equine oocytes
311 (Hinrichs 2010a). The finding that cCOCs exhibit higher glucose consumption than eCOCs may agree with the
312 "quiet embryo theory" developed by Leese (2002) who suggested that cell viability is associated with a 'quiet'
313 metabolism. His theory proposed that the most viable cells exhibit a 'quieter' metabolism by regulation of gene
314 expression and changes in the transcriptome and proteome, as they are required to use less energy (Baumann et al.
315 2007). Thus, as cCOCs experience a higher degeneration rate, it could be plausible that the higher metabolic rate
316 observed is related to the cCOCs' attempts to maintain their structures and functions but this hypothesis needs to
317 be further explored.
318 Due to the marked differences found in the parameters studied between expanded and compact specimens, in the
319 next experimental set the relative mRNA expression of genes related to glycolysis, apoptosis and meiotic
320 competence was studied (Table 1). Surprisingly, no differences were observed in the expression of *GDF9* and
321 *BMP15* between eCOCs and cCOCs despite their divergent meiotic competence (Fig. 2). The only developmental
322 competence marker that showed a significant higher expression in eCOCs and EC compared to their compact
323 counterparts was *TNFAIP6*. This gene has been previously described in the equine species and its expression is
324 enhanced by gonadotropins during follicular maturation and ovulation (Sayasith *et al.* 2007; Sayasith *et al.* 2013)
325 and depends upon the mare´s age (Sessions-Bresnahan and Carnevale 2015). The extracellular matrix of equine
326 follicles, as well as COCs, expresses *TNFAIP6* which has been related to cumulus expansion and extrusion of the
327 detached complex during ovulation in the horse (Sayasith *et al.* 2007) and influences the meiotic competence of
328 porcine oocytes (Yuan *et al.* 2011). Although *GDF9* and *BMP15* have been described to be differently expressed
329 in oocytes of young vs. aged mares (Campos-Chillon *et al.* 2015) and mature and immature oocytes, (Scarlet *et al.*
330 2017) no previous reports have been published regarding a possible differential expression in eCOCs and cCOCs.
331 It is widely known that *GDF9* and *BMP15* are involved in cumulus expansion and are related to enhanced meiotic
332 competence (Kidder and Vanderhyden 2010). However, in the horse GDF9 and BMP15 are expressed in the
333 oocyte and not in the cumulus cells (Scarlet et al. 2017) and their expression varies depending upon the oocyte´s

meiosis stage in swine oocytes (Lin *et al.* 2014). In view of these data, our results demonstrate that *TNAFIP6* could be more reliable than *GDF9* or *BMP15* to predict complete cumulus expansion as well as the meiotic competence of pooled equine COCs at different meiotic stages.

When the relative abundance of the glycolysis related genes *PFKP*, *PDK3,LDHA* and glucose transporters 1 and 3 (encoded by *SLC2A1* and *SLC2A3*) was compared between expanded and compact mural granulosa cells, only *SLC2A1* exhibited a significantly increased expression in EC (Fig. 3). The expression of glucose transporter 1*(SLC2A1)* has been shown to dramatically increase in dominant follicles, thecal and granulosa cells undergoing atresia in bovine (Nishimoto *et al.* 2006); in addition, negative correlations between glucose concentrations in follicular fluid and glucose transporter 1 and 3 expression have also been reported (Nishimoto *et al.* 2006). This scenario has been described in glucose-deprived bovine chromaffin cells in which *SLC2A1* and *SLC2A3* were singificantly overexpressed (Fladeby *et al.* 2003), implying a local regulatory mechanism of glucose uptake to compensate glucose deprivation. Coincidentally, one of the first signs of follicluar atresia is the loss of thecal vascularity and a decrease in the glucose and oxygen supply to the follicles. As previously stated, EE are retrieved from atretic follicles in the horse explaining the higher *SLC2A1* expression and lower glucose consumption compared to CC, which are obtained from juvenile follicles (Hinrichs and Schmidt 2000). The higher expression of apoptosis related genes in CC are in agreement with previous reports in goat oocytes in which granulosa cell expansion inversely correlates with apoptosis after IVM (Han *et al.* 2006). The higher relative abundance of apoptotic-related mRNAs and lower expression of *TNFAIP6* in CC may indicate an incomplete or less-functional expansion explaining why many cCOCs degenerate after IVM (Table 2 and Fig. 2).

In conclusion our results demonstrate that compact COCs and mural granulosa cells exhibit higher glucose consumption and lower meiotic competence compared to their expanded counterparts. The relative abundance of *SLC2A1* and *TNFAIP6* is consistently higher in expanded mural granulosa cells while the expression of apoptotic related genes predominates in compact mural granulosa cells. *TNFAIP6* is highly expressed in expanded samples (COCs and mural cells) and could be a more reliable predictor of complete granulosa cell expansion and meiotic competence than *GDF9* and/or *BMP15* in equine pooled COCs. More research is needed to establish if, in order to increase the meiotic competence of equine COCs, the IVM conditions should vary for compact and expanded specimens in view of the vivid metabolic differences observed.

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Figure legends

Fig. 1 Glucose consumption in compact cumulus oocyte complexes (cCOCs); expanded cumulus oocyte complexes (eCOCs); compact mural granulosa cells (CC) and expanded mural granulosa cells (EC). Glucose consumption (1A); pyruvate (1B) and lactate production (1C) by cCOCs, eCOCs, CC and EC. For this experiment 116 COCs were used: 17 cCOCs and 99 eCOCs. For COCs, values are expressed as nmol/COC; for mural cells values are expressed as pmol/µg protein. Data are presented as the mean percentage ± SEM. A student t-test was used to compare a pair of values; statistical significance was set at * $p < 0.05$.

Fig. 2 Relative mRNA expression of genes related to oocyte's meiotic competence, apoptosis and glucose metabolism in compact cumulus oocyte complexes (cCOCs) and expanded cumulus oocyte complexes (eCOCs). The relative abundance of the candidate genes related to meiotic competence (*TNFAIP6*, *GDF9*, *BMP15*), apoptosis (*FAS*,*BAX*, *BCL2L1*) and glucose metabolism (*SCL2A1*, *SCL2A3*, *PFKP*,*PDK3*, *LDHA*) was compared between cCOCs and eCOCs after IVM. Bars represent the mean of 5 different replicates and the error bar show the standard error of the mean. A student t-test or Mann-Whitney Rank Sum Test was used to compare pairs of values; statistical significance was set at * $p < 0.05$.

577 **Fig. 3** Relative mRNA expression of genes related to oocyte's meiotic competence, apoptosis and glucose
578 metabolism in compact mural granulosa (CC) and expanded mural granulosa (EC).The relative mRNA expression
579 of the candidate genes related to oocyte competence (*TNFAIP6*), apoptosis (*FAS*, *FASLG*,*BAX*, *BCL2L1*) and
580 glucose metabolism (*SCL2A1*, *SCL2A3*, *PFKP*,*PDK3*, *LDHA*) in CC and EC was compared after IVM. Bars
581 represent the mean of 3 different replicates and the error bar shows the standard error of the mean. A student t-test
582 or Mann-Whitney Rank Sum Test was used to compare pair of values; statistical significance was set at * $p < 0.05$.

583

584 **Suppl. File 1** Classification of equine COCs obtained by follicular scraping. Equine follicles were scraped and the
585 cells present in the Petri dish as well as the COCs were carefully evaluated. If any signs of expansion were found
586 in the dish or cumulus, the oocytes were classified as expanded. A) compact COC; B) and C) expanded COCs.
587 The micrographs shown were obtained at 20 x.

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Table 1. Gene symbols, accession numbers, primer sequences and amplicon product length for the target genes examined

Entrez gene symbol	Gene name	Accession no.	Primer sequence (5′–3′)
BAX	BCL2 associated X, apoptosis regulator	XM_014729721.1	Forward:CCAGGATGCGTCCACCAAGAAG Reverse:GCCCACCTTGAGCACCAATTTG
BMP15	Bone morphogenetic protein 15	XM_001496223.2	Forward:GTGAAGCCCTTGACCAATGT Reverse:AGGTGAAGTTGATGGCGGTA
FAS	Tumour necrosis factor receptor superfamily, member 6	XM_005602380.1	Forward:TTACGTGCAAACATGGGATCA Reverse:TCCGGATCCTTCTCTGCATT
FASLG	Fas ligand	NM_001166039.1	Forward:GCTGGTTGTTGCAGGACTGA Reverse:TCAATGACACCGGGCTGTAC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001163856	Forward:ATGGTGAAGGTCGGAGTAAAC Reverse:TGTAGACCATGTAGTTGAGGTCA
GDF9	Growth differentiation factor 9	XM_001504427.2	Forward:GACTTGAGTAAGTGTTCCACAGCA Reverse:CAGAGGCCACCTCTACAACAC
H2AFZ	H2A histone family, member Z	NM_174809	Forward:AGGACGACTAGCCATGGACGTGTG Reverse:CCACCACCAGCAATTGTAGCCTTG
LDHA	Lactate dehydrogenase A	NM_001144880.1	Forward:CCGTGTTATCGGAAGTGGTTGC Reverse:AGAATCTCCATGCTCCCCAAGG
PDK3	Pyruvate dehydrogenase kinase, isozyme 3	XM_005614047.1	Forward:CAGAGAACCCAGAGATGCTTCA Reverse:AGCTTAGAATCCTGGGGAGGT
PFKP	Phosphofructokinase, platelet	XM_005606965.1	Forward:AAGAACGTGCTGGGCCACATG Reverse:TTCGTGGTGATCCATTGCATGG
SLC2A1	Solute carrier family 2 member 1	NM_001163971.1	Forward:CACTGGAGTCATCAACGCCC Reverse:CCACGATCAGCATCTCAAAG
RN18S	*18S*rRNA	NR_046271.1	Forward:AGAAACGGCTACCACATCCAA Reverse:CCTGTATTGTTATTTTTCGTCACTACCT
SLC2A3	Solute carrier family 2 member 3	XM_001498757.2	Forward:GGAGACCACCACCAATGTCTAAGC Reverse:TCCTGGGTTACAACCTGATGAGG
TNFAIP6	Tumour necrosis factor alpha induced protein 6	NM_001081906.1	Forward:GAAGGCGGTGTGTGAATACGA Reverse:GGCTTCACAATGGGGTATCCA

Table 2. Chromatin configuration after IVM

Values show the total number of oocytes in each group, with percentages in parentheses. Within columns, values with different superscript letters differ significantly (P <0.001). GV, germinal vesicle; DEG,degenerated

Oocyte type	GV (%)	MI (%)	MII (%)	DEG (%)	Total no. oocytes
Compact oocytes	5 (10.9)	3 (6.5)	10 (21.7)[a]	28 (60.9)[a]	46
Expanded oocytes	21 (10.9)	10(5.2)	96 (50.0)[b]	65 (33.9)[b]	192

Figure 1A

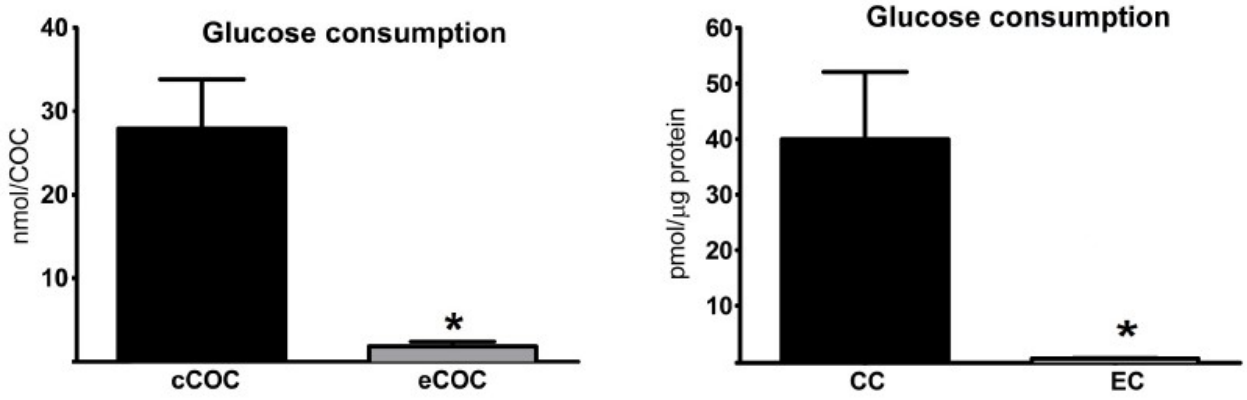

Figure 1B

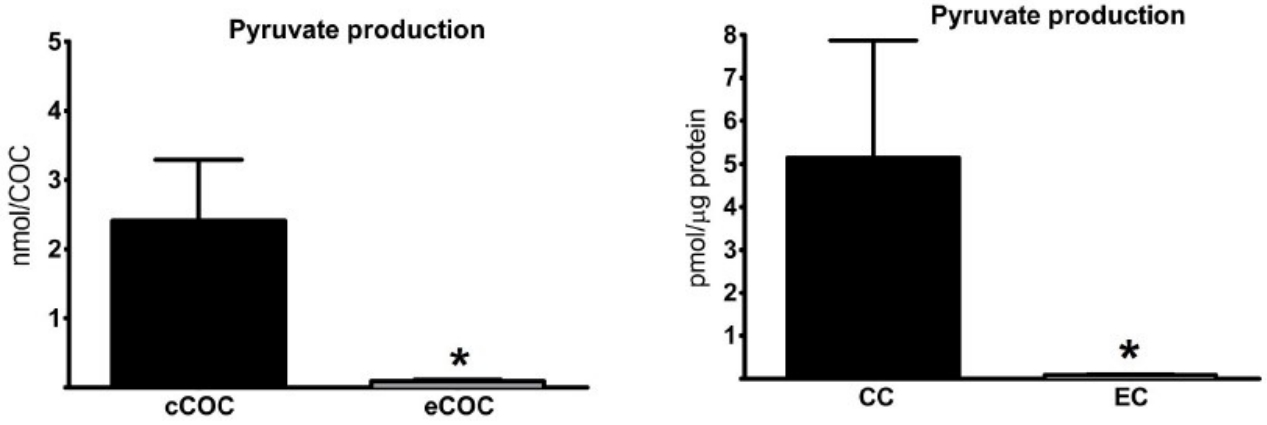

Figure 1C

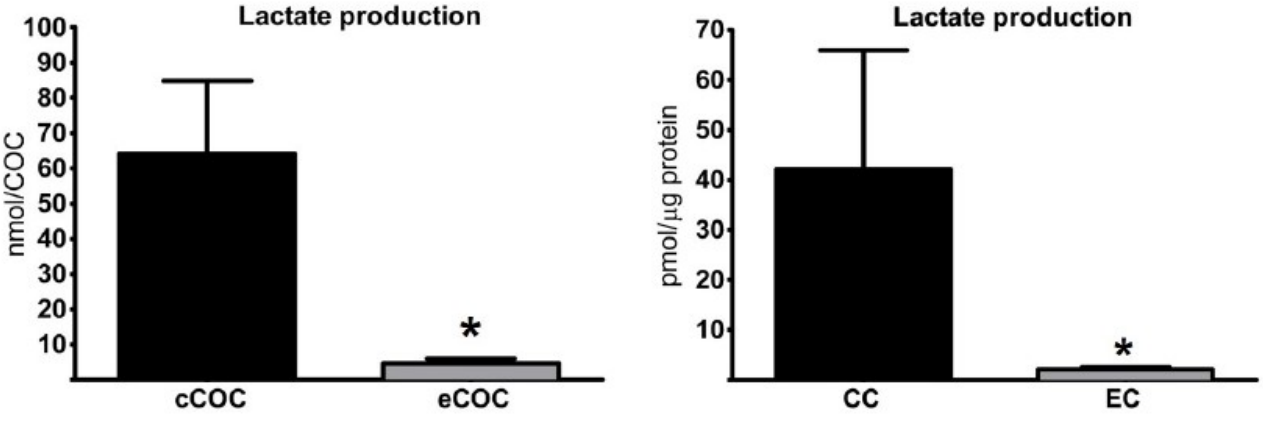

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Figure 2

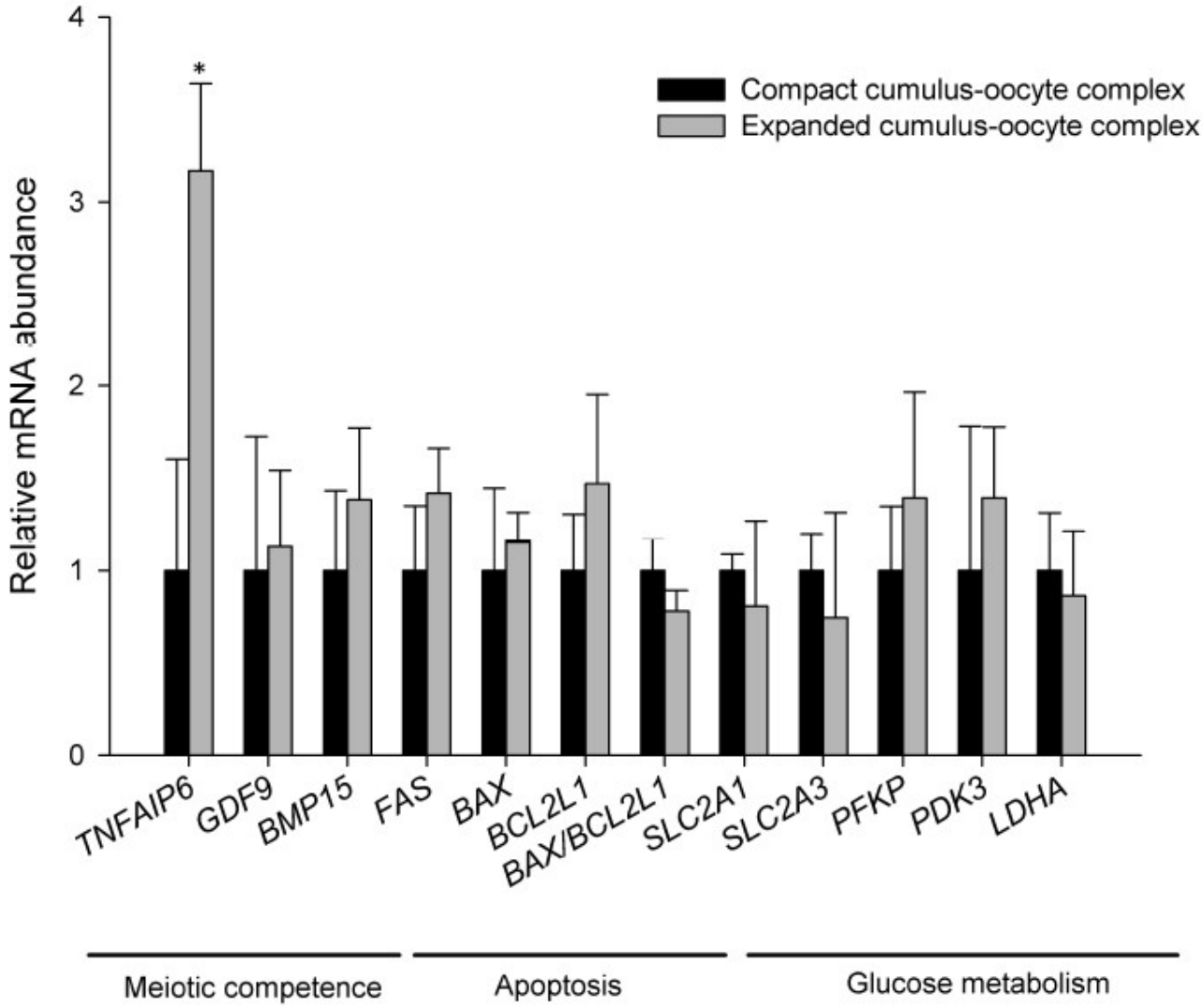
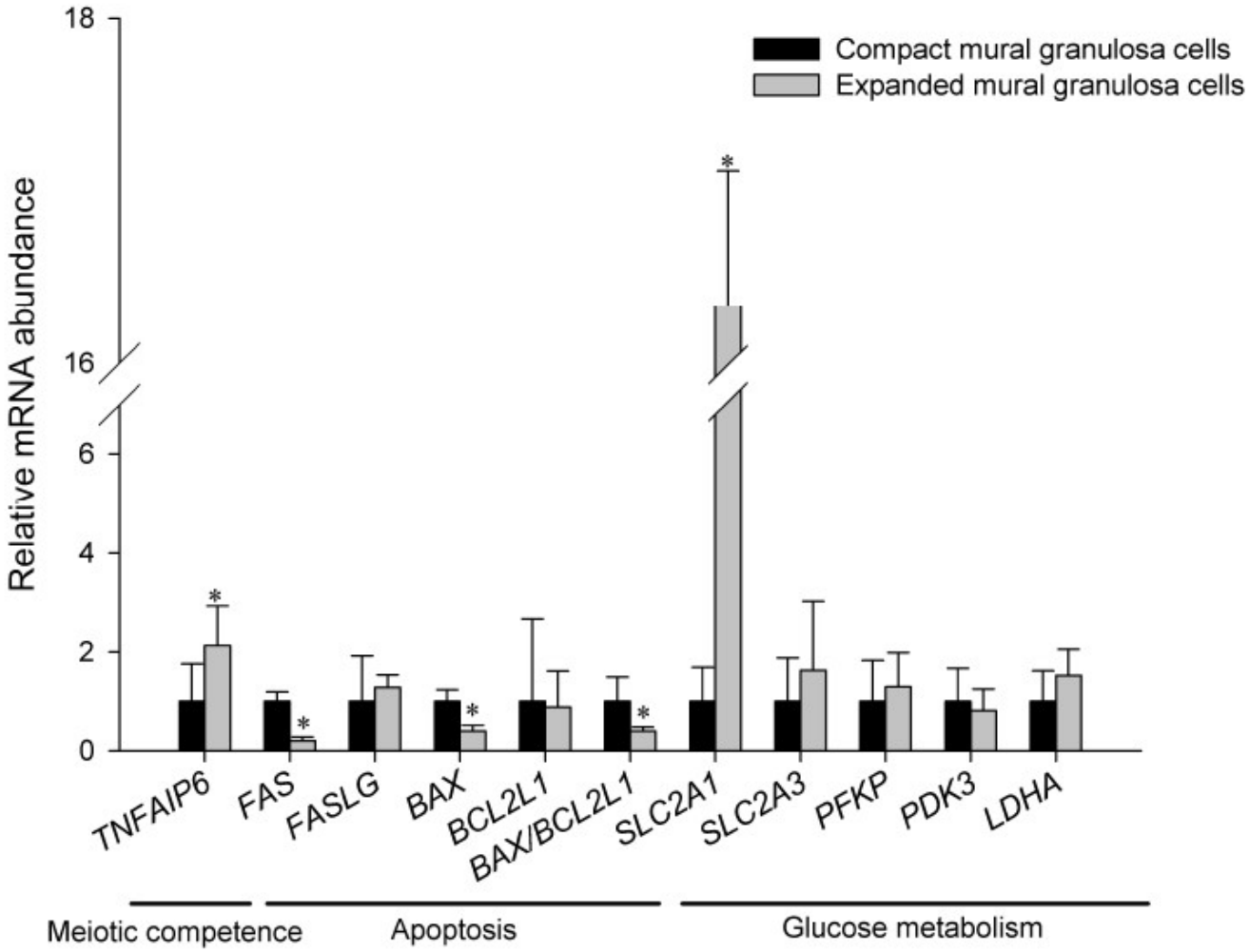

Relative mRNA abundance
4
3
2
1
0
*
Compact cumulus-oocyte complex
Expanded cumulus-oocyte complex
TNFAIP6
GDF9
BMP15
FAS
BAX
BCL2L1
BAX/BCL2L1
SLC2A1
SLC2A3
PFKP
PDK3
LDHA
Meiotic competence
Apoptosis
Glucose metabolism

Figure 3


Compact mural granulosa cells
Expanded mural granulosa cells
Relative mRNA abundance
18
16
6
4
2
0
*
TNFAIP6
FAS
FASLG
BAX
BCL2L1
BAX/BCL2L1
SLC2A1
SLC2A3
PFKP
PDK3
LDHA
Meiotic competence
Apoptosis
Glucose metabolism

Suppl. File 1