In Vitro Cellular & Developmental Biology - Animal

Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin

--Manuscript Draft--

Manuscript Number:	
Full Title:	Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin
Article Type:	Articles (full research papers)
Keywords:	porcine oocyte maturation; fatty acids; gas chromatography; trans-10, cis-12 conjugated linoleic acid; forskolin
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Abstract:	The effect of maturation and of two lipid modulators supplementation along in vitro maturation (IVM) on fatty acid (FA) and dimethylacetal (DMA) composition of porcine cumulus oocyte complexes (COC) were studied. Abattoir derived immature COC were analyzed for FA and DMA or submitted to IVM as follows: control group; t10,c12 CLA group, t10,c12 CLA supplementation during 44h; Forskolin group, forskolin supplementation during the initial 2h; t10,c12 CLA+forskolin group, t10,c12 CLA during 44h and forskolin just 2h. Each experimental group had 5 replicates. FA analysis of oocytes, cumulus cells (CC), follicular fluid and culture media were performed by gas-liquid chromatography. Oocytes and their CC had different FA composition. Oocytes were richer in saturated FA (SFA) preferentially maintaining their FA profile during maturation. Mature CC had the highest polyunsaturated FA (PUFA) content. Five individual and total SFA, and monounsaturated FA (MUFA), notably oleic acid (c9-18:1), percentages were lower (P≤0.023) in mature than in immature CC. t10,c12 CLA was accumulated by COC from t10,c12 CLA and t10,c12 CLA+forskolin groups, mostly in CC where MUFA and a eicosatrienoic isomer decreased (P≤0.043). Nevertheless PUFA or FA and DMA total content were not affected. Arachidonic acid was reduced in t10,c12 CLA+forskolin CC and hexadecanal-DMA in t10,c12 CLA CC. Forskolin alone increased (P≤0.043) c9-18:1 in oocytes. In conclusion, maturation process clearly changed porcine COC FA and DMA profiles, mostly of CC, also more susceptible to modifications induced by t10,c12 CLA. This possibility of manipulating COC lipid

	composition during IVM could be used to improve oocyte quality/cryopreservation efficiency.

Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin

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Abstract

The effect of maturation and of two lipid modulators supplementation along in vitro maturation (IVM) on fatty acid (FA) and dimethylacetal (DMA) composition of porcine cumulus oocyte complexes (COC) were studied. Abattoir derived immature COC were analyzed for FA and DMA or submitted to IVM as follows: control group; *t*10,*c*12 CLA group, *t*10,*c*12 CLA supplementation during 44h; Forskolin group, forskolin supplementation during the initial 2h; *t*10,*c*12 CLA+forskolin group, *t*10,*c*12 CLA during 44h and forskolin just 2h. Each experimental group had 5 replicates. FA analysis of oocytes, cumulus cells (CC), follicular fluid and culture media were performed by gas-liquid chromatography. Oocytes and their CC had different FA composition. Oocytes were richer in saturated FA (SFA) preferentially maintaining their FA profile during maturation. Mature CC had the highest polyunsaturated FA (PUFA) content. Five individual and total SFA, and monounsaturated FA (MUFA), notably oleic acid (*c*9-18:1), percentages were lower ($P \leq 0.023$) in mature than in immature CC. *t*10,*c*12 CLA was accumulated by COC from *t*10,*c*12 CLA and *t*10,*c*12 CLA+forskolin groups, mostly in CC where MUFA and a eicosatrienoic isomer decreased ($P \leq 0.043$). Nevertheless PUFA or FA and DMA total content were not affected. Arachidonic acid was reduced in *t*10,*c*12 CLA+forskolin CC and hexadecanal-DMA in *t*10,*c*12 CLA CC. Forskolin alone increased ($P \leq 0.043$) *c*9-18:1 in oocytes. In conclusion, maturation process clearly changed porcine COC FA and DMA profiles, mostly of CC, also more susceptible to modifications induced by *t*10,*c*12 CLA. This possibility of manipulating COC lipid composition during IVM could be used to improve oocyte quality/cryopreservation efficiency.

Key words: porcine oocyte maturation, fatty acids, gas chromatography, trans-10, cis-12 conjugated linoleic acid, forskolin.

Introduction

The bi-directional exchanges between the oocyte and the surrounding cumulus cells (CC) are crucial for the acquisition of oocyte developmental competence (Ouandaogo *et al*., 2011). Nonetheless in terms of lipid metabolism the knowledge of this cross talk is scarce. Previous reports demonstrated that mammalian oocytes contain a lipid reserve mainly constituted of triacylglycerols and membrane structural phospholipids. Furthermore, the abundance and utilization of this oocyte lipid reserve seems to be species-specific. Undoubtedly porcine oocytes exhibit the highest fatty acid (FA) content among domestic animals (McEvoy *et al.*, 2000; Fujihira *et al.*, 2004), but information about their CC FA composition and function remains obscure.

According to Sturmey and Leese (2003) and Sturmey *et al*. (2006) the intracellular lipids of porcine oocytes are available for energy supply during maturation, but uptake of exogenous lipids from culture media enriched with follicular fluid or serum may also occur (Kim *et al*., 2001; Sturmey *et al*., 2009). Therefore, exogenous FA may be metabolized or accumulate in the ooplasm changing oocyte lipid content and FA composition (Sturmey *et al*., 2009) and probably those of CC. Besides the metabolic role of FA as energy source, lipids play an essential role in cell structure and functions namely as intracellular messengers. Moreover being the major components of cell membranes they have particular relevance in oocyte-spermatozoon interaction (Gardner and Evans, 2006) and in cell resistance to cryopreservation (Kim *et al.*, 2001; Arav and Zvi, 2008; Pereira and Marques, 2008). Thus the clarification of COC lipid composition and contribution to oocyte metabolism during maturation is of primordial importance.

Several studies have reported that triacylglycerols and phospholipids of the porcine reproductive tissues are notably rich in polyunsaturated fatty acids (PUFA), particularly

those of the graafian follicles and ovaries (Holman and Hofstetter, 1964) and also of the immature oocytes (Homa *et al.*, 1986; McEvoy *et al.*, 2000). However, in terms of qualitative and quantitative analysis of FA and lipid content, data on porcine oocytes are still limited. To our knowledge the FA and other membrane constituents such as ether vinylic chains from plasmalogens phospholipids [firstly identified by Homa *et al*. (1986) in immature oocyte and currently analysed as dimethylacetals – DMA] composition of mature porcine oocytes have not yet been reported. Additionally it has not been established whether storage and lipid metabolism influence oocyte maturation and its developmental potential as well as the role of CC along the entire process. Furthermore the possibility of modifying intracellular lipids of oocytes/embryos by changing the composition of the culture medium to increase their quality and cryotolerance is still under investigation (Pereira and Marques, 2008; Sturmey *et al.*, 2009).

Recently, *t*10,*c*12 CLA and forskolin (FSK) both recognized as intracellular lipid modulators were shown to improve porcine and bovine embryos cryosurvival (Men *et al.*, 2006; Pereira *et al.*, 2007; 2008). During maturation, this conjugated dienoic FA interfered on the FA profile of bovine oocytes improving their competence to develop into higher quality embryos (Lapa *et al.*, 2011). Moreover porcine oocytes treated with 10 µM FSK for the initial 2h or 100 µM *t*10,*c*12 CLA for the entire period of in vitro maturation (IVM), presented modifications on the distribution and morphology of cytoplasmic lipid droplets (Prates *et al.*, 2012). At the doses tested, while oocytes supplemented with *t*10,*c*12 CLA exhibited a lighter cytoplasmic colour tone, oocytes exposed to FSK had smaller lipid droplets. These findings led to the hypothesis that *t*10,*c*12 CLA and/or FSK supplementation during porcine oocyte maturation might be used to change the lipid content and FA composition of these gametes.

The objectives of the present study were firstly to characterize the FA and DMA content and composition of porcine oocytes and CC before and after maturation. Secondly we investigated if *t*10,*c*12 CLA and/or FSK culture media supplementation might change the FA and DMA composition and maturation rates of porcine COC.

Materials and Methods

Experimental design

The effect of IVM and of the supplementation of maturation media of porcine oocytes with *t*10,*c*12 CLA (Matreya, Pleasent Gap, PA, USA; ref. 001249; 100 μM) (Prates *et al.*, 2012) and/or with FSK (10 μM) (Men *et al.*, 2006) on the FA and DMA content and composition of COC were investigated in five replicates as described below.

To study the FA and DMA content and composition of porcine COC during maturation, COC were separated in CC and oocytes and both evaluated as immature and mature. Therefore four groups were considered: 1) immature oocytes, 2) immature CC, 3) mature oocytes and 4) mature CC. The FA and DMA composition of follicular fluid from the pool of ovaries and of the pre-IVM media used in each session were also analysed.

To investigate the effect of *t*10,*c*12 CLA and FSK on porcine COC maturation four experimental groups were constituted: control group, COC were matured during 44h without supplementation; (2) *t*10,*c*12 CLA group, COC were matured during 44h supplemented with 100 μM *t*10,*c*12 CLA, (3) FSK group- COC were supplemented with 10 μM FSK during the initial 2h of IVM; (4) *t*10,*c*12 CLA+FSK group - COC were matured with 100 μM *t*10,*c*12 CLA during 44h plus10 μM FSK during the first 2h of maturation. At the end of the maturation period, samples of mature oocytes were used for lipid content analysis, while others (control group n=84; *t*10,*c*12 CLA group n=87; FSK

group n=78; *t*10,*c*12 CLA+FSK group n=85) were fixed and stained for individual evaluation of maturation rate. As above in each session for FA and DMA composition analysis, COC from all treatments were divided in CC and oocytes and both evaluated as control, *t*10,*c*12 CLA, FSK and *t*10,*c*12 CLA+FSK, resulting in 10 experimental groups. After COC maturation, the FA and DMA content and composition were also assessed in IVM culture media.

Reagents and culture media

Otherwise stated, all products were purchased from Sigma-Aldrich (Sintra, Portugal). The medium used for the collection and washing of COC was the Dulbecco's phosphate-buffered saline (DPBS) composed of 4mg/mL bovine serum albumin (BSA; fraction V), 0.34 mmol/L sodium pyruvate, 5.4 mmol/L D-glucose and 70 µg/mL kanamycin (mDPBS) (Gil *et al.*, 2005). Oocyte maturation medium was the BSA-free North Carolina State University (NCSU23) (Petters and Weels, 1993), supplemented with 10% (v/v) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor and 100 µmol/L glutathione.

Cumulus oocyte complexes collection

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported at 38 ºC in a 0.9% NaCl solution containing 70 µg/mL kanamycin. At the laboratory, the ovaries were washed several times in mDPBS. Follicles of 3–6 mm in diameter were aspirated and the follicular content placed in a conical flask. The supernatant was centrifuged (3700 g during 30min) and the resultant follicular fluid retrieved for FA analysis. The remaining COC were washed in mDPBS. Oocytes with a compact cumulus

mass and a dark and evenly granulated cytoplasm were selected for maturation rates assessment or FA analysis.

In vitro maturation

Selected oocytes were washed and placed in 3 mL of maturation medium, supplemented with 10 IU/mL equine chorionic gonadotrophin (Folligon; Intervet International, Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotrophin (Veterin Corion; Divasa Farmavic, Barcelona, Spain), during 22h and then for another 22h in maturation medium without hormones. Oocyte maturation (100-250 oocytes per 3 mL) was performed at 39 ºC in a humidified atmosphere of 5% CO_2 in air.

Assessment of oocyte maturation

At the end of the maturation period, CC were stripped off the oocytes by vortexing in a 1% sodium citrate solution. Denuded oocytes were fixed for 3–4 days in aceto/ethanol (1:3, v:v) then stained with 1% aceto/lacmoid solution. Oocytes were classified as matured when metaphase II (MII) chromosomes set and the chromatin of the first polar body were present. Fixed oocytes with abnormal appearance were discharged.

Fatty acids and dimethylacetal analysis

For lipid extraction, CC were removed from COC before and after maturation, by vortexing for up to 10 min. Then denuded oocytes and CC were washed three times in 1% sodium citrate. Cell concentration was determined using a haemocytometer camera. In each session, immature and mature oocytes (110 to 200 per pooled group sample) and their CC (7×10^4 to 2×10^6 per pooled group sample) were suspended in citrate solution (5 replicates or sessions in total). Also, 500 µL of culture media before and after maturation, of 1% sodium citrate solution and of follicular fluid from the pool of ovaries used in each

session were collected. Then, fatty acid methyl esters (FAME) were prepared by direct transesterification. Briefly, 1 mL of toluene and 200 µL of 19:0 (0.5 mg/mL) as an internal standard were added to each sample in fresh. After the addition of 2 mL of sodium methoxide in methanol (0.5 M), the solution was vortexed and left for about 15min at 50 ºC. After cooling to room temperature, 3 mL of 10% HCl solution was allowed to react for more 30min at 80 ºC. Once cooled, 6% potassium carbonate was added in two portions of 2 mL to prevent excessive effervescence, followed by addition of 2 mL of hexane. The solution was vortexed, centrifuged and the organic layer transferred to another tube. The extraction step was repeated twice. The final solution was dried using anhydrous sodium sulphate.

The solvent was then removed under a stream of nitrogen and the final residue dissolved in 100 µL *n*-hexane (GC grade > 99%). The resultant FAME were injected (2 µL), via a Hewlett Packard (Avondale, PA, USA) HP 6890 Series GC System, onto a CP-Sil 88 fused-silica capillary column (100 m length, 0.25 mm internal diameter, 0.20 µm film thickness: Chromopack, Varian Inc., Walnut Creek, CA, USA). Injection was performed using a programmed temperature and a splitless method. The injector and detector temperatures were 250 ºC and 280 ºC, respectively. Initial oven temperature of 45 ºC was held for 5min, increased at 10 ºC to 175 ºC and held for 30min and increased at 50 ºC/min to 215 ºC and held for 35min. Helium was used as carrier gas at a flow rate of 1 mL/min. Peaks were identified by comparison with a commercial standard FAME mix 4:0 to 24:0 purchased from Supelco Inc. (Bellefonte, PA, USA) and a bacterial acid methyl esters mix purchased from Matreya LLC (Pleasant Gap, PA, USA). Quantities of each FA present were calculated by reference to internal standard. Additionally, identification of some unknown peaks and confirmation of FAME were performed by GLC-MS using a Varian

Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column.

Statistical Analysis

All results are expressed as least square means±standard error. Data representing 5 replicates of oocyte nuclear configuration as well as FA and DMA composition of COC and culture media were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). For FA and DMA composition evaluation of immature and mature COC, the model included the effect of maturation status and cell type (oocytes and CC) and its interaction. For FA and DMA composition evaluation of mature COC treated with *t*10,*c*12 CLA, FSK and FSK+*t*10,*c*12 CLA, the model included the treatment, cell type (oocytes and CC) and its interaction. The cell type was treated as repeated measures within COC assuming a compound symmetry covariance matrix. Homogeneity of variance between groups was tested and when necessary the group statement was included to accommodate variance heterogeneity. The model was simplified when some variable were not detected in one cell type or maturation status. Orthogonal contrasts comparing each treatment with the control were computed. When interactions were significant ($P \leq 0.05$) differences between means were determined by the PDIFF multiple comparison procedure. The mixed linear model used for oocyte nuclear configuration and FA and DMA culture media composition included treatment as fixed effect and session as random effect. Differences were considered significant when $P \leq 0.05$.

Results

The FA and DMA composition of porcine follicular fluid and pre-IVM medium analyzed by gas-liquid chromatography is described in table 1. The most abundant FA, in decreasing order, were palmitic (16:0), oleic (*c*9-18:1), linoleic (18:2n-6), stearic (18:0) and arachidonic (20:4n-6) acids. The FA composition of follicular fluid and maturation media is notably similar, with the major difference being the absence of adrenic acid (22:4n-6) in pre-IVM medium.

Effect of the maturation status on the FA and DMA composition of porcine COC

The FA and DMA composition of COC prior to and after IVM is presented in table 2. Both oocytes and CC tended (P=0.056) to increase total FA and DMA content during maturation (immature oocytes=51.1±4.10 *vs*. mature oocytes=63.7±4.10 ng per oocyte; immature CC=0.013±0.010 *vs*. mature CC=0.032±0.010 ng per CC). Differences in FA profile between cell type (C), maturation status (MS) as well as differential effects of maturation status in each cell type (MS×C interaction) were observed (table 2). Oocytes presented higher 16:0 (P<0.001) and hexadecenoic (*c*7-16:1, P=0.031) acids, but lower 18:0 (P=0.001), hexadecanal (DMA-16:0, P=0.004) and octadecanal (DMA-18:0, P=0.006) DMAs proportions than CC. In vitro maturation increased (P=0.018) the 18:2n-6 proportion in both oocytes and CC. For the majority of FA displaying a MS×C interaction (myristic, 14:0; pentadecyclic, 15:0; margaric, 17:0; *c*9-18:1; arachidic, 20:0; tricosylic, 23:0; and docosadienoic, 22:2n-6) the general trend was that maturation induced a decrease in its proportions on CC but not in oocytes. The exception was the 20:4n-6 that increased during maturation in CC. Considering the partial sums of FA, the maturation process reduced SFA and MUFA in CC and increased PUFA, whereas the oocytes remained unaffected.

Several lipid compounds were not detected at all in immature or mature oocytes and immature CC (eicosatrienoic, 20:3^{niI}; clupanodocnic, 22:5n-3; and octadecenal-DMA, DMA-18:1). Others, were not detected only in immature oocytes and immature CC (margaric, *c*9-17:1; and γ-linolenic, 18:3n-6) or displayed only an inconsistent occurrence in immature oocytes samples (α-linolenic, 18:3n-3; gondoic, *c*11-20:1, 20:3^{niII}; eicosadienoic, 20:2n-6; 22:4n-6; and cervonic, 22:6n-3). In those cases, although data available for immature oocytes were displayed in table 2, the statistical analysis, when possible, involved only the comparison of mature oocytes and CC. Thus, mature CC contained higher proportion of 22:6n-3 and an unidentified 20:3 isomer but lower 22:4n-6 than mature oocytes. Two 20:3 isomers (herein designated as 20:3^{niI} and 20:3^{niII}) were detected in the porcine oocytes and CC. From gas-liquid chromatography-mass spectrometry (GLC-MS) confirmation, they were identified as 20:3 FA however their structures were not completely elucidated. Nevertheless, it was clear from coelution with reference standards, that these 20:3 isomers were not the common 20:3n-6, 20:3n-3 and 20:3n-9 isomers.

Effect of t10,c12 CLA and FSK on oocyte maturation and FA and DMA composition of porcine COC and culture media

In this study the effect of the supplementation of porcine oocyte culture medium with *t*10,*c*12 CLA, FSK or both on oocyte maturation and FA and DMA composition of COC and culture media after 44h of IVM were evaluated by the observation of oocyte nuclear configuration and gas-liquid chromatography respectively. These supplementations did not interfere (P>0.05) with oocyte maturation rates (MII: control group=87.99±0.04%, *t*10,*c*12 CLA group=84.71±0.04%, FSK group=86.94±0.04%, *t*10,*c*12 CLA+FSK group=84.98±0.04%) or FA and DMA content expressed as ng/ individual cell (table 3).

Nonetheless differences were identified on the FA and DMA profiles of COC and culture media.

The most expressive differences in FA composition are between types of cells (oocytes and CC). Independently of treatment, mature oocytes presented higher proportions of saturated (14:0, 15:0, 16:0 but not 18:0) and monounsaturated (*c*7-16:1; palmitoleic, *c*9-16:1; vaccenic, *c*11-18:1 and *c*11-20:1, but not *c*9-18:1) FA than mature CC. Conversely, CC contained higher proportions of 18:0 and most of PUFA (18:2n-6, 20:2n-6, 20:4n-6, 22:6n-3) as well as DMA-16:0 and DMA-18:0. Consistently, mature oocytes presented higher SFA and lower PUFA than mature CC. The *c*9-18:1 was higher in CC than in oocytes in all treatments except on *t*10,*c*12 CLA treatment where was strongly reduced in CC to levels observed in oocytes. Moreover, *t*10,*c*12 CLA treatment seems to drastically eliminate the DMA-18:0 from mature CC, which was not detected only in CC cells treated with *t*10,*c*12 CLA. This effect seems to be reversed when FSK was also included in the maturation medium. On the contrary of other groups (P=0.053), the 20:4n-6 proportion was equal in CC and oocytes of *t*10,*c*12 CLA+FSK group. Forskolin inclusion in maturation media has almost no effect on CC FA profile but slightly reduced the 15:0 (P=0.010) and raised the *c*9-18:1 in oocytes (P=0.043).

Independently of cell type, the CLA treatment reduced (P<0.05) the proportions of DMA-16:0, 18:3n-6 and *c*11-20:1 and tended (P<0.10) to reduce *c*7-16:1, *c*9-16:1, *c*11-18:1 and 20:4n-6. When *t*10,*c*12 CLA was included in the maturation medium with FSK (*t*10,*c*12 CLA+FSK) the proportions of 18:3n-6, 20:4n-6, 22:5n-3 and DMA-16 were also reduced.

The FA and DMA composition of culture media after COC maturation from all treatments is described in table 4. The *t*10,*c*12 CLA was only detected when it was supplemented to the culture media. Lower concentrations of *c*9-18:1 (P=0.022) and *c*11-18:1 (P=0.004) and consequently of total MUFA (P=0.007) were observed in *t*10,*c*12 CLA group than in

289 control or FSK groups. However, in the *t*10,*c*12 CLA and *t*10,*c*12 CLA+FSK
290 supplemented media the percentages of 22:6n-3 were higher (P=0.022) than in control.
291
292 **Discussion**
293
294 Porcine oocytes are for long known to be highly rich in lipids although their nature and
295 function have been largely overlooked (Homa *et al.*, 1986; Sturmey *et al.*, 2006 and 2009).
296 The present study provides new insights on FA and DMA composition of porcine COC
297 prior to and during IVM. Results showed for the first time that immature oocytes and their
298 CC have different FA profiles which change differentially along maturation. While oocytes
299 are notably rich in SFA, preferentially maintaining their FA profile, immature CC are
300 characterized by an abundant MUFA content and their FA profile is greatly modified
301 during maturation. In mature CC, SFA and MUFA are reduced while PUFA content is
302 higher and diverse. However either in oocytes or in CC the FA and DMA content tends to
303 increase along maturation. Moreover, *t*10,*c*12 CLA and/or FSK supplementation
304 influenced COC and culture media FA and DMA composition but their total contents were
305 not modified.
306 Emerging studies in the field of developmental biology revealed the extremely intricate
307 COC as a complete functional and dynamic unit. The bi-directional exchanges between the
308 oocyte and the surrounding CC are critical for the acquisition of oocyte competence, CC
309 expansion and early embryonic development. Moreover this cross-talk between CC and
310 oocyte plays a pivotal role in oocyte metabolism during maturation (Sutton *et al.*, 2003;
311 Gilchrist and Thompson, 2007; Ouandaogo *et al.*, 2011). In porcine oocytes this process is
312 long and requires different metabolites for energy production (Sturmey and Leese, 2003).
313 Since these gametes cannot use glucose directly, CC catabolise glucose by the glycolytic

314 pathway, supplying pyruvate or lactate to the oocyte as oxidative substrates (Sutton *et al*.,
315 2003). A metabolic role for triacylglycerols providing ATP through FA mitochondrial β-
316 oxidation which is essential for protein synthesis along cytoplasmic maturation and
317 meiosis was also suggested by Sturmey and Leese (2003) and Sturmey *et al.* (2006). These
318 authors showed that the triacylglycerol content of immature oocytes decreases during
319 maturation with sufficient O_2 consumption to be accounted for this depletion. Additionally
320 Romek *et al*. (2011), also through an indirect method of lipid evaluation using lipid
321 droplets staining, reported that *in vivo* total lipid content in immature porcine oocytes was
322 about 21% higher than in mature. On the contrary, our results determined by internal
323 standard gas-chromatography quantification of FA, showed that FA content of COC,
324 oocytes and CC, tend (P=0.056) to increase with the maturation process. As herein
325 McEvoy *et al*. (2000) reported that the most abundant FA in immature porcine oocytes was
326 the 16:0, followed by the *c*9-18:1, 18:0, 18:2n-6 and 20:4n-6, although the amount of total
327 FA was lower in the present study. This FA profile was maintained after IVM. Thus in
328 order to support maturation, the oocytes ought to accumulate but also to metabolize their
329 constituent FA and/or utilize those supplied by the CC or from the culture medium
330 enriched with follicular fluid.
331 Previously it was reported by Cetica *et al*. (2002) that during bovine oocyte maturation, CC
332 may supply FA to the oocytes for further participation on their metabolism. In addition
333 these oocytes were able to perform a selective FA uptake from culture medium during
334 maturation (Kim *et al.*, 2001; Lapa *et al.*, 2011) to maintain their intracellular FA reserves.
335 Eventually FA elongation or *de novo* synthesis may also occur. These mechanisms might
336 also take place during porcine COC maturation. In fact the FA profile of CC changed
337 drastically along this process. The SFA, in particular 14:0, 15:0, 17:0, 20:0, 23:0, as well as
338 MUFA, notably *c*9-18:1, were reduced in mature CC. Therefore it is possible that during

maturation porcine CC can either extensively metabolize SFA and MUFA and/or supply them to the oocyte. Moreover the proportion of mature CC PUFA, composed by eleven individual FA (table 2), was the highest of all COC constituents. The majority of these long chain FA as well as the DMA derived from the vinyl ether chains of plasmalogens are components of membrane phospholipids (Homa *et al.*, 1986; McEvoy *et al.*, 2000). Thus porcine CC might undergo an extensive intracellular remodeling associated with its metabolic differentiation resulting in increased amounts of internal membrane.

On the other hand, oocytes are found to be highly chilling sensitive due to a characteristic high ratio of SFA to PUFA (Arav and Zvi, 2008). This ratio results from the existence of a selective protective process to ensure that PUFA composition of oocytes is kept in a safe minimum level to avoid the risk of cellular damage (Sturmey *et al.*, 2009; Lapa *et al.*, 2011). In accordance, porcine oocytes are mainly constituted by SFA preserving their FA profile during maturation. Interestingly, although oocytes were cultured in a PUFA containing media, mainly 18:2n-6, they did not uptake provisions of all these available FA. The most abundant FA in porcine follicular fluid and thus in IVM media was the 16:0. This SFA may be used by porcine oocytes having a beneficiary effect on their quality (Warzych *et al.*, 2011), in contrast to its harmful action in bovine oocytes (Van Hoeck *et al.*, 2011). Nonetheless when supplemented with *t*10,*c*12 CLA porcine oocytes incorporate this PUFA but in much lower amounts than in CC. Despite that, PUFA percentage was not changed neither in *t*10,*c*12 CLA oocytes nor in CC suggesting that *t*10,*c*12 CLA replaced other PUFA.

Several studies reported the activity of lipid modulators namely *t*10,*c*12 CLA and FSK during oocyte maturation (Lapa *et al.*, 2011; Fu *et al.*, 2011) or embryonic development (Men *et al.*, 2006; Pereira *et al.*, 2007; 2008). According to Prates *et al.* (2012) during porcine oocyte maturation FSK impaired meiosis beyond 2h of supplementation while

364 *t*10,*c*12 CLA had no effect on meiotic progression. Moreover both substances interfered
365 with the distribution and morphology of lipid droplets. However if these morphological
366 modifications of IVM porcine oocytes (Fu *et al.* 2011, Prates *et al*., 2012) reflect changes
367 of their FA composition was not investigated. Herein we found differences in FA and also
368 in DMA composition of COC supplemented with *t*10,*c*12 CLA, FSK or both. This isomer
369 was accumulated by COC from *t*10,*c*12 CLA and *t*10,*c*12 CLA+FSK groups, mostly in CC
370 where MUFA and a 20:3 isomer decreased ($P \leq 0.043$) though PUFA, total FA and DMA
371 contents were not affected. Thus in the present conditions, the identified *t*10,*c*12 CLA
372 activity in reducing lipid accumulation (Pariza, 2001; Pereira *et al.*, 2007; 2008) was not
373 demonstrated for porcine oocytes. Equally although FSK can induce lipolysis in porcine
374 embryo cells and oocytes reducing their lipid content (Men *et al.*, 2006; Fu *et al.*, 2011),
375 we could not confirm this reduction, since the FA and DMA contents were not different
376 from control in FSK mature oocytes. Nevertheless, when FSK was supplemented alone for
377 2h, the *c*9-18:1 increased in mature oocytes.
378 Although *t*10,*c*12 CLA and FSK did not interfere on nuclear meiosis progression, a
379 possible additive effect of these substances on 20:4n-6 metabolization by CC, probably
380 through eicosanoid cascade (Nuttinck *et al*., 2008, Lapa *et al.*, 2011), seems to occur. In
381 fact during maturation the 20:4n-6 decreases 3.85% in *t*10,*c*12 CLA+FSK CC when
382 compared to control (P=0.023). Both products can affect the protein kinase A and cAMP
383 signaling pathways (Men *et al*., 2006; Ashwell *et al*., 2010) thus exerting a synergic effect.
384 Interestingly, CC of porcine COC treated with *t*10,*c*12 CLA revealed more modifications
385 in the FA profile than those of bovine where only 20:4n-6 were reduced in mature oocytes
386 but not in CC (Lapa *et al.*, 2011). These divergences may be related to differences in COC
387 acquisition of developmental competence between these species. Furthermore, herein both
388 DMA-16:0 and DMA-18:0 were higher in CC than in oocytes from all groups. However

389 *t*10,*c*12 CLA treatment reduced the DMA-16:0 in COC and the DMA-18:0 (which became
390 undetectable) from CC of *t*10,*c*12 CLA group. These effects of *t*10,*c*12 CLA seem to be
391 mitigated when FSK was also present. A decrease in plasmalogens of cellular membranes
392 might interfere with their dynamics and functions (Nagan *et al.*, 2001). The membranes
393 composition can therefore interfere in the communication between oocytes and CC or CC
394 adherence looseness during CC expansion, being crucial for appropriate oocyte maturation
395 and fertilization (Sutton *et al.*, 2003; Gilchrist and Thompson, 2007). The importance of
396 plasmalogens in porcine COC maturation requires further investigation.
397
398 **Conclusions**
399 In conclusion, the findings from the present study indicate that 16:0, 18:0, *c*9-18:1, 18:2n-6
400 and 20:4n-6 are the major constituents of porcine follicular fluid and COC although in
401 different orders of abundance. Moreover it highlights the distinct FA and DMA profiles of
402 oocytes and CC during maturation. Both immature and mature porcine oocytes are rich in
403 SFA preferentially maintaining their profile as well as their SFA and PUFA ratio during
404 maturation probably to prevent the significant risk of free radical damage currently
405 associated to FA unsaturations. The marked variation in FA profile of CC during this
406 process could indicate a major participation in supplying substrates to the oocyte and an
407 extensive intracellular remodeling resulting in increased amounts of internal membrane
408 and consequently an enrichment in PUFA and DMA at the end of maturation. Besides CC
409 PUFA reservoir, mostly 18:2n-6 and 20:4n-6 might be involved in FA elongation or PG
410 synthesis. On the other hand, porcine COC can uptake and accumulate *t*10,*c*12 CLA from
411 the culture media, being CC more susceptible to FA and DMA modifications induced by
412 this CLA isomer than oocytes whereas FSK during 2h induced an increase of *c*9-18:1 in
413 oocytes. Therefore, data of FA and DMA composition of porcine COC suggest a pivotal

role of both cumulus cells and oocytes in lipid metabolism through maturation. This information can complete previous morphological evaluations and should be used to enhance oocyte quality and the efficiency of *in vitro* embryo production and cryopreservation.

Acknowledgments

The "Fundação para a Ciência e Tecnologia" is greatly acknowledged for funding a grant for E.G. Prates (SFRH/BD/42359/2007).

References

Arav A, Zvi R **(**2008) Do chilling injury and heat stress share the same mechanism of injury in oocytes? *Mol Cell Endocrinol* 282: 150–152.

Ashwell MS, Ceddia RP, House RL, Cassady JP, Eisena EJ, Eling TE, Collins JB, Grissom SF, Odle J (2010) Trans-10, cis-12-conjugated linoleic acid alters hepatic gene expression in a polygenic obese line of mice displaying hepatic lipidosis. *J Nutr Biochem* 21: 848–855.

Cetica P, Pintos L, Dalvit G, Beconi M (2002) Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation *in vitro*. *Reproduction* 124: 675–681.

Fu X, Wu G, Li G, Hou Y, Zhou G, Wang Y, Zhu S (2011) Positive effects of forskolin (stimulator of lipolysis) treatment on cryosurvival of *in vitro* matured porcine oocytes. *Theriogenology* 75: 268–275.

437 Fujihira T, Kinoshita M, Sasaki M, Ohnishi M, Ishikawa H, Ohsumi S, Fukui Y (2004)
438 Comparative studies on lipid analysis and ultrastructure in porcine and southern minke
439 whale (Balaenoptera Bonaerensis) oocytes. *J Reprod Dev* 50: 525-532.
440 Gardner AJ, Evans JP (2006) Mammalian membrane block to polyspermy: new insights
441 into how mammalian eggs prevent fertilisation by multiple sperm. *Reprod Fertil Dev* 18:
442 53-61.
443 Gil MA, Roca J, Cremades T, Hernández M, Vázquez JM, Rodríguez-Martínez, Martínez
444 EA (2005) Does multivariate analysis of post-thaw sperm characteristics accurately
445 estimate in vitro fertility of boar individual ejaculates? *Theriogenology* 64: 305-316.
446 Gilchrist RB, Thompson JG **(**2007) Oocyte maturation: Emerging concepts and
447 technologies to improve developmental potential *in vitro*. *Theriogenology* 67: 6–15
448 Holman RT, Hofstetter HH **(**1964) The fatty acid composition of the lipids from bovine
449 and porcine reproductive tissues. *J Am Oil Chem Soc* 42: 540-544.
450 Homa ST, Racowsky C, McGaughey RW (1986) Lipid analysis of immature pig oocytes. *J*
451 *Reprod Fertil* 77: 425-434.
452 Kim JY, Kinoshita M, Ohnishi M, Fukui Y (2001) Lipid and fatty acid analysis of fresh
453 and frozen-thawed immature an *in vitro* matured bovine oocytes. *Reproduction* 122: 131–
454 138.
455 Lapa M, Marques CC, Alves SP, Vasques MI, Baptista MC, Carvalhais I, Silva Pereira M,
456 Horta AEM, Bessa RJB, Pereira RM (2011) Effect of trans-10, cis-12 conjugated linoleic
457 acid on bovine oocyte competence and fatty acid composition. *Reprod Dom Anim* 46:
458 904–910.
459 McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speak BK (2000**)** Fatty acid
460 composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida.
461 *J Reprod Fertil* 118: 163–170.

462 Men H, Agca Y, Riley LK, Critser JK (2006) Improved survival of vitrified porcine
463 embryos after partial delipation through chemically stimulated lipolysis and inhibition of
464 apoptosis. *Theriogenology* 66: 2008-2016.
465 Nagan N, Zoeller RA (2001) Plasmalogens: biosynthesis and functions. *Prog Lipid Res*
466 40: 199-229.
467 Nuttinck F, Guienne BM, Clément L, Reinaud P, Charpigny G, Grimard B (2008)
468 Expression of genes involved in prostaglandin E_2 and progesterone production in bovine
469 cumulus–oocyte complexes during *in vitro* maturation and fertilization. *Reproduction* 13:
470 593–603.
471 Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, De Vos J, Hamamah S 2011.
472 Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage.
473 *PLoS ONE 6*: e27179.
474 Pariza MH, Park Y, Cook ME (2001) The biologically active isomers of conjugated
475 linoleic acid. *Prog Lipid Res* 40: 283–298.
476 Pereira RM, Marques CC (2008) Animal oocyte and embryo cryopreservation. Cell Tissue
477 Bank **9** 267–277.
478 Pereira RM, Baptista MC, Vasques MI, Horta AEM, Portugal PV, Bessa RJB, Chagas e
479 Silva J, Silva Pereira M, Marques CC (2007**)** Cryosurvival of bovine blastocysts is
480 enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t,12c CLA). *Anim*
481 *Reprod Sci* 98: 293–301.
482 Pereira RM, Carvalhais I, Pimenta J, Baptista MC, Vasques MI, Horta AEM, Santos IC,
483 Marques MR, Reis A, Silva Pereira M, Marques CC (2008) Biopsied and vitrified bovine
484 embryos viability is improved by trans-10, cis-12 conjugated linoleic acid supplementation
485 during *in vitro* embryo culture. *Anim Reprod Sci* 106: 322-332.

Prates EG, Marques CC, Baptista MC, Vasques MI, Carolino N, Horta AEM, Charneca R, Nunes JT, Pereira RM (2012). Fat area and lipid droplets morphology of porcine oocytes during *in vitro* maturation with trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. *Animal* (in press).

Petters RM, Wells KD (1993) Culture of pig embryos. *J Reprod Fertil* 48: 61–73.

Romek M, Gajda B, Krzysztofowicz E, Kepczynski M, Smorag Z (2011) New technique to quantify the lipid composition of lipid droplets in porcine oocytes and pre-implantation embryos using Nile Red fluorescent probe. *Theriogenology* 75: 42-54.

Sturmey RG, Leese HJ (2003) Energy metabolism in pig oocytes and early embryos. *Reproduction* 126: 197–204.

Sturmey RG, Toole PJO, Leese HJ (2006) Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction* 132: 829–837.

Sturmey RG, Reis A, Leese HJ, McEvoy TG (2009) Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Dom Anim* 44: 50–58.

Sutton ML, Gilchrist RB, Thompson JG (2003) Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 9: 35-48.

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ. Bols PEJ, Leroy JLMR (2011) Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLoS ONE 6*: e23183.

Warzych E, Cieslak A, Pawlak P, Renska N, Pers-Kamczyc E, Lechniak D (2011) Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes. *Vet Med (Praha)* 56: 156–167.

Table 1. Fatty acid and dimethylacetal (DMA) composition of porcine follicular fluid and maturation media prior to culture (pre-IVM medium). Data are least square means (LSM)±standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

Name	Formula	Follicular fluid	Pre-IVM medium
Myristic	14:0	0.56±0.038	0.58±0.067
Myristoleic	*c*9-14:1	0.053±0.010	0.210±0.042
Pentadecyclic	15:0	0.24±0.016	0.27±0.039
Palmitic	16:0	24.1±0.40	22.4±0.60
Hexadecenoic	*c*7-16:1	1.02±0.074	0.87±0.045
Palmitoleic	*c*9-16:1	1.27±0.58	1.08±0.115
Heptadecanoic	17:0	0.71±0.098	0.75±0.040
Margaric	*c*9-17:1	0.31±0.067	0.25±0.011
Stearic	18:0	13.4±0.50	14.7±0.50
Oleic	*c*9-18:1	19.4 ± 0.62	20.8±0.39
Cis-Vaccenic	*c*11-18:1	2.37±0.206	1.97±0.104
Linoleic	18:2n-6	17.3±1.12	19.4±0.70
Arachidic	20:0	0.24± 0.045	0.38±0.050
γ-Linolenic	18:3n-6	0.42±0.059	0.30±0.026
α-Linolenic	18:3n-3	0.32 ± 0.048	0.28±0.016
Gondoic	*c*11-20:1	0.20±0.022	0.15±0.018
Eicosadienoic	20:2n-6	0.37±0.066	0.29±0.037
Eicosatrienoic^{I}	20:3^{niI}	0.62 ± 0.050	0.57±0.091
Eicosatrienoic^{II}	20:3 ^{niII}	0.76 ± 0.087	0.56±0.022
Arachidonic	20:4n-6	11.6±0.56	10.4±0.31
Tricosylic	23:0	0.05±0.012	0.23±0.055
Docosadienoic	22:2n-6	0.08±0.032	0.23±0.014
Adrenic	22:4n-6	0.33±0.036	nd
Clupanodonic	22:5n-3	1.34±0.048	1.11±0.078
Cervonic	22:6n-3	0.73±0.089	0.44±0.061
Other FA	Other FA	0.30±0.031	0.33±0.087
Dimethylacetal	**DMA**		
Hexadecanal-	DMA-16:0	0.87±0.062	0.57±0.063
Octadecanal-	DMA-18:0	0.77±0.111	0.57±0.049
Octadecenal-	DMA-18:1	0.34±0.049	0.38±0.045
Partial sums			
SFA		39.3±0.61	39.3±0.68
MUFA		24.9±0.95	25.6±0.50
PUFA		33.8±0.56	33.5±0.69
n6/n3 ratio		12.7±0.70	16.9 ± 1.08

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detectable; ni^{I} and ^{II}, non identified 20:3 isomers.

Table

Table 2. Effect of the maturation status (immature vs. mature, MS) on fatty acid and dimethylacetal (DMA) composition of porcine oocytes and cumulus cells. Data are least square means (LSM)±standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

	Immature		Mature		*P values*		
	Oocytes	Cumulus cells	Oocytes	Cumulus cells	MS	Cell type (C)	MS x C
14:0	1.66^{ab}±0.529	1.75^{ab}±0.529	2.11^{b}±0.101	1.06^{a}±0.101	0.832	0.004	0.001
15:0	0.57^{ab}±0.126	0.98^{b}±0.126	0.80^{b}±0.045	0.43^{a}±0.045	0.161	0.865	0.004
16:0	44.9±1.81	24.1±0.74	45.2±1.06	26.4±1.20	0.365	<0.001	0.434
*c*7-16:1	1.50±0.097	0.96±0.069	1.95±0.295	1.45±0.216	0.072	0.031	0.898
*c*9-16:1	1.30±0.047	1.33±0.120	1.58±0.117	1.27±0.152	0.390	0.258	0.190
17:0	1.30^{a}±0.258	2.64^{b}±0.258	1.10^{a}±0.147	0.86^{a}±0.147	0.036	0.002	<0.001
*c*9-17:1	nd	nd	0.29±0.031	0.23±0.016	-	0.150	-
18:0	11.6±0.86	15.6±0.86	10.7±0.33	13.4±0.33	0.093	0.001	0.324
*c*9-18:1	17.0^{ab}±0.43	29.2^{c}±1.45	16.5^{a}±0.43	20.4^{b}±1.45	0.012	<0.001	0.005
*c*11-18:1	3.08±0.286	2.19±0.278	2.82±0.119	2.62±0.331	0.765	0.074	0.228
18:2n-6	5.18±0.323	6.59±0.304	6.24±0.277	8.31±0.501	0.018	0.001	0.387
20:0	0.87^{a}±0.333	2.43^{b}±0.333	1.10^{a}±0.333	0.49^{a}±0.333	0.067	0.180	0.010
18:3n-6	nd	nd	0.73±0.206	0.90±0.103	-	0.507	-
18:3n-3	0.19±0.019*	nd	0.29±0.049	0.20±0.021	-	0.221	-
*c*11-20:1	0.30±0.009*	nd	0.29±0.099	0.39±0.042	-	0.438	-
20:2n-6	0.23±0.030*	nd	0.10±0.186	0.71±0.078	-	0.093	-
20:3^{niI}	nd	nd	nd	0.42±0022	-	-	-
20:3^{niII}	0.69±0.078*	nd	0.43±0.114	0.94±0.066	-	0.018	-
20:4n-6	5.95^{a}±0.768	5.71^{a}±0.768	5.28^{a}±0.444	13.4^{b}±0.44	0.004	<0.001	<0.001
23:0	0.70^{a}±0.234	1.74^{b}±0.234	0.50^{a}±0.062	0.47^{a}±0.062	0.030	0.001	0.001
22:2n-6	1.09^{a}±0.177	3.34^{b}±0.594	0.75^{a}±0.177	0.54^{a}±0.594	0.023	0.048	0.023
22:4n-6	0.68±0.113*	nd	0.70±0.043	0.45±0.025	-	0.007	-
22:5n-3	nd	nd	nd	1.00±0.051	-	-	-
22:6n-3	0.29±0.035*	nd	0.28±0.032	0.45±0.019	-	0.011	-
Other FA	nd	nd	nd	0.60±0.126	-	-	-
DMA							
DMA-16:0	0.71±0.123	0.98±0.123	0.41±0.123	1.27±0.123	0.972	0.004	0.072
DMA-18:0	0.70±0.198	1.12±0.216	0.37±0.134	0.79±0.123	0.219	0.006	0.985
DMA-18:1	nd	nd	nd	0.51±0.027	-	-	-
Partial Sums							
SFA	61.6^{c}±1.45	49.2^{b}±1.45	61.3^{c}±1.45	43.1^{a}±1.45	0.116	<0.001	0.049
MUFA	23.0^{a}±1.09	33.5^{c}±1.09	23.3^{a}±1.09	27.0^{b}±1.09	0.050	<0.001	0.013
PUFA	14.0^{a} ± 0.50	15.7^{a} ± 1.17	14.6^{a} ± 0.50	27.4^{b} ± 1.17	0.002	<0.001	<0.001

Data within the same row with different superscripts letters are statistically different due to an interaction between cell type and maturation status ($P \leq 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, MS, maturation status; C, cell type ; nd, not detectable; ni^{I} and ^{II}, non identified 20:3 isomers; § data not included in statistical analysis;

Table

Table 3. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA), forskolin (FSK) or both (*t*10,*c*12 CLA+FSK) supplementation during in vitro maturation on fatty acid and dimethylacetal (DMA) composition of mature porcine oocytes and cumulus cells. Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

	Control		*t*10,*c*12 CLA		FSK		*t*10,*c*12 CLA+FSK		*P values*	
	Oocytes	Cumulus cells	Oocytes	Cumulus cells	Oocytes	Cumulus cells	Oocytes	Cumulus cells	C	T x C
14:0	2.11±0.195	1.06±0.424	1.93±0.195	1.23±0.424	1.95±0.195	1.99±0.424	2.33±0.195	1.49±0.242	0.015	0.402
15:0	0.80^{c}±0.059	0.43^{a} ±0.042	0.73^{bc} ±0.046	0.43^{a} ±0.063	0.60^{b} ±0.013	0.58^{ab} ±0.063	0.65^{bc} ±0.113	0.53^{ab} ±0.093	<0.001	0.010
16:0	45.2±1.43	26.4±0.05	44.5±1.97	25.84±0.66	45.9±1.63	25.9±0.63	44.5±0.98	25. 0±0.50	<0.001	0.926
*c*7-16:1	1.95±0.233	1.46±0.104	1.83±0.233	0.91±0.104	1.94±0.233	1.16±0.104	1.97±0.233	0.95±0.119	<0.001	0.514
*c*9-16:1[1,3]	1.58±0.088	1.27±0.088	1.39±0.088	1.00±0.088	1.54±0.088	1.22±0.088	1.47±0.088	0.83±0.088	<0.001	0.355
17:0	1.17±0.063	0.93±0.078	1.07±0.063	1.17±0.211	1.11±0.108	1.42±0.151	1.04±0.043	1.33±0.287	0.288	0.070
*c*9-17:1	0.27±0.023	0.23±0.032	0.24±0.017*	nd	0.15±0.020	0.25±0.036	0.24±0.020	0.23±0.042	0.537	0.085
18:0	10.7±0.37	13.4±2.05	9.98±0.365	17.6±2.05	9.80±0.365	15.4±2.05	10.3±0.37	15.1±2.05	<0.001	0.432
*c*9-18:1	16.5^{a}±0.34	20.4^{cd}±0.97	17.4^{ab}±0.34	18.2^{bc}±0.97	17.8^{b}±0.34	22.4^{d}±0.97	16.8^{ab}±0.34	21.8^{d}±0.97	<0.001	0.043
*c*11-18:1[3]	2.82±0.230	2.62±0.175	2.57±0.127	1.99±0.273	2.77±0.158	2.16±0.211	2.67±0.163	1.90±0.235	0.002	0.557
18:2n-6	6.24±0.343	8.31±0.538	6.13±0.058	8.74±0.619	6.09±0.173	7.56±0.300	5.97±0.143	8.47±0.384	<0.001	0.231
20:0	1.10±0.328	0.49±0.095	0.59±0.139	0.60±0.112	0.69±0.026	1.15±0.192	0.75±0.058	1.13±0.282	0.655	0.067
18:3n-6[1,3]	0.74±0.193	0.90±0.171	0.35±0.038	0.32±0.007	0.49±0.134	0.76±0.118	0.29±0.064	0.42±0.143	0.163	0.310
18:3n-3	0.32±0.031	0.20±0.024	0.24±0.024	0.22±0.024	0.20±0.027	0.27±0.031	0.24±0.027	0.25±0.027	0.438	0.084
*c*11-20:1[1]	0.29±0.030	0.39±0.026	0.31±0.026	0.27±0.026	0.29±0.026	0.42±0.030	0.30±0.026	0.36±0.027	0.025	0.137
*t*10,*c*12 CLA	nd	nd	1.17±0.294	6.46±2.053	nd	nd	1.25±0.294	6.29±2.053	0.008	0.932
20:2n-6	0.27±0.030	0.71±0.133	0.25±0.021	0.58±0.133	0.19±0.025	0.59±0.149	0.25±0.021	0.56±0.133	<0.001	0.900
20:4n-6[3]	5.28^{a}±0.340	13.4^{c}±0.50	5.18^{a}±0.198	10.3^{c}±1.46	5.44^{a}±0.218	11.4^{c}±0.89	5.04^{a}±0.410	9.55^{b}±1.441	<0.001	0.053
20:3 ni[I 2]	nd	0.42±0.143	nd	0.38±0.143	nd	0.97±0.143	nd	0.56±0.143	-	-
20:3 ni[II]	0.43^{a}±0.106	0.94^{b}±0.103	0.74^{ab}±0.171	0.55^{a}±0.071	0.58^{ab}±0.140	1.00^{b}±0.091	0.75^{ab}±0.122	0.49^{a}±0.067	0.149	0.004
23:0[2]	0.50±0.108	0.47±0.108	0.69±0.108	0.61±0.108	0.62±0.108	0.91±0.123	0.91±0.058*	nd	0.496	0.246
22:2n-6	0.75±0.212	0.54±0.235	0.79±0.212	0.56±0.235	0.57±0.212	1.13±0.266	1.04±0.212	1.08±0.235	0.804	0.330

22:4n-6	0.70±0.056	0.45±0.034*	0.73±0.056	nd	0.64±0.056	nd	0.74±0.056	nd	-	-
22:5n-3[3]	nd	1.00±0.051	nd	1.00±0.051	nd	0.88±0.051	nd	0.77±0.051	-	-
22:6n-3	0.28±0.023	0.45±0.029	0.27±0.019	0.41±0.050	0.27±0.024	0.43±0.018	0.31±0.023	0.37±0.018	<0.001	0.137
DMA										
DMA-16:0[1,3]	0.41±0.050	1.27±0.119	0.37±0.050	0.74±0.119	0.46±0.050	1.02±0.119	0.42±0.050	0.82±0.119	<0.001	0.05
DMA-18:0	0.38±0.079	0.79±0.069	0.24±0.203*	nd	0.27±0.092	0.63±0.069	0.44±0.080	0.63±0.080	<0.001	0.351
DMA-18:1[1]	nd	0.51±0.027	nd	0.25±0.027	nd	nd	nd	nd	-	-
Partial sums										
SFA	61.3±0.99	43.1±1.06	59.3±0.56	47.3±4.95	60.5±0.31	46.7±0.77	59.9±0.61	44.3±2.21	<0.001	0.05
MUFA[1]	23.3^{ab} ±0.60	27.0^{c} ±1.05	24.4^{ab} ±0.60	22.4^{a} ±1.05	24.5^{ab} ±0.60	27.4^{c} ±1.05	23.4^{ab} ±0.60	25.7^{bc} ±1.05	0.012	0.017
PUFA	14.6±0.35	27.4±2.32	15.8±0.35	29.5±2.32	14.4±0.35	24.3±2.32	15.8±0.35	28.6±2.32	<0.001	0.696
FA (ng/un)[III]	63.7±6.12	0.03±0.011	65.6±6.12	0.04±0.011	62.7±6.12	0.01±0.011	69.1±6.12	0.02±0.011	<0.001	0.887

Data in the same line with different superscript letters are significantly different ($P \leq 0.05$) due to an interaction between cell type and treatment; different superscript numbers (1 to 3) represent the contrasts of each treatment against the control: 1 – contrast for t10c12CLA treatment against control ($P \leq 0.05$), 2 – contrast for FSK treatment against control ($P \leq 0.05$), 3 – contrast for t10c12CLA+FSK against control ($P \leq 0.05$)C, cell type; T, treatment; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, nd, not detectable; * data not included in statistical analysis; [I] and [II] – non identified 20:3 isomers; [III] – total FA content per individual cell.

Table

Table 4. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA), forskolin (FSK) or both (*t*10,*c*12 CLA+FSK) supplementation during in vitro maturation of porcine cumulus oocyte complexes on fatty acid and dimethylacetal (DMA) composition of culture media. Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

	Culture media				*P*
	Control	*t*10,*c*12 CLA	FSK	*t*10,*c*12 CLA+FSK	*values*
14:0	0.82±0.029	0.74±0.075	0.78±0.030	0.78±0.022	0.682
*c*9-14:1	0.30±0.054	0.27±0.075	0.43±0.063	0.46±0.075	0.241
15:0	0.31±0.024	0.24±0.024	0.31±0.024	0.31±0.028	0.140
16:0	21.9±0.38	21.5±0.38	22.3±0.38	21.8±0.38	0.542
*c*7-16:1	0.94±0.051	1.03±0.051	0.98±0.051	0.83±0.059	0.131
*c*9-16:1	1.22±0.051	1.23±0.051	1.20±0.051	1.05±0.058	0.142
17:0	0.86±0.078	0.77±0.078	0.72±0.078	0.75±0.078	0.653
*c*9-17:1	0.244±0.014	0.22±0.014	0.24±0.014	0.23±0.016	0.713
18:0	13.9±0.59	14.7±0.59	13.4±0.59	14.0±0.59	0.495
*c*9-18:1	21.0^{b}±0.35	19.4^{a} ±0.35	21.0^{b}±0.35	20.2^{ab}±0.35	0.022
*c*11-18:1	2.07^{b}±0.090	1.56^{a}±0.090	1.97^{b}±0.090	2.16^{b}±0.104	0.004
18:2n-6	19.8±0.53	19.2±0.53	20.3±0.53	19.3±0.53	0.441
20:0	0.41±0.080	0.51±0.080	0.44±0.080	0.42±0.092	0.820
18:3n-6	0.41±0.029	0.41±0.029	0.44±0.029	0.40±0.033	0.775
18:3n-3	0.28±0.039	0.29±0.039	0.32±0.039	0.37±0.45	0.466
*c*11-20:1	0.13±0.357	0.13±0.357	0.20±0.357	0.88±0.357	0.410
*t*10,*c*12 CLA	nd	2.74 ± 1.017	nd	2.35 ±1.017	0.797
20:2n-6	0.24±0.079	0.37±0.079	0.34±0.079	0.27±0.091	0.608
$20:3^{niI}$	0.50±0.027	0.47±0.027	0.46±0.027	0.46±0.032	0.756
$20:3^{niII}$	0.50±0.021	0.43±0.021	0.47±0.021	0.51±0.024	0.120
20:4n-6	9.84±0.249	9.89±0.249	10.4±0.25	9.97±0.25	0.396
23:0	0.34±0.078	0.45±0.078	0.31±0.078	0.42±0.090	0.602
22:2n-6	0.24±0.457	0.45±0.457	0.29±0.457	0.22±0.457	0.428
22:4n-6	0.22±0.119	0.34±0.119	nd	nd	0.520
22:5n-3	1.09±0.177	1.15±0.177	1.13±0.177	1.62±0.177	0.167
22:6n-3	0.43^{a} ±0.025	0.56^{b}±0.025	0.50^{ab}±0.025	0.54^{b}±0.029	0.022
Other FA	0.41 ± 0.059*	nd	nd	nd	
DMA					
DMA-16:0	0.84±0.103	0.59±0.103	0.64±0.103	0.59±0.119	0.328
DMA-18:0	0.56±0.050	0.56±0.069	0.50±0.050	0.51±0.058	0.806
DMA-18:1	0.38±0.032	0.28±0.046	0.22±0.046	0.26±0.046	0.083
Partial sums					
SFA	38.4 ±0.97	38.8±0.97	38.2±0.97	37.8±0.97	0.891
MUFA	26.3^{c}±0.45	23.7^{a}±0.45	25.9^{bc}±0.45	24.7^{ab}±0.45	0.007
PUFA	33.6 ±0.96	36.3±0.96	34.7±0.96	36.4±0.96	0.159

Data within the same row with different superscripts letters are statistically different ($P \leq 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detectable; ni^{I} and ^{II}, non identified 20:3 isomers.

Editor of In vitro Cellular &Developmental Biology- Animal

Vale de Santarém, January 2nd, 2013

Dear Sir

Please find enclosed a manuscript entitled "Fatty acid composition of porcine cumulus oocyte complexes during maturation: effect of the lipid modulators *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin " that we wish to publish in In vitro Cellular &Developmental Biology - Animal.

Porcine oocytes are for long known to be highly rich in lipids although their nature and function has been largely overlooked. The present study provides new insights on fatty acid (FA) and dimethyl-acetal (DMA) composition of porcine cumulus-oocyte complexes (COC) prior to and during in vitro maturation. Moreover the effect of two lipid modulators, the *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin, on this composition was studied. Our results showed for the first time that the maturation process differentially affected the FA and DMA composition of oocytes and their cumulus cells. While oocytes are notably rich in saturated FA, preferentially maintaining their FA profile, immature cumulus cells are characterized by an abundant MUFA content and their FA profile is greatly modified during maturation. This marked variation in the FA profile of cumulus cells could indicate a major participation in supplying substrates to the oocyte and an extensive intracellular remodeling resulting in increased amounts of internal membrane and consequently an enrichment in PUFA and DMA at the end of maturation. Moreover, *t*10,*c*12 CLA and/or forskolin supplementation influenced porcine oocyte COC and culture media FA and DMA composition. This possibility of manipulating lipid composition of COC during maturation could be used as a tool to improve oocyte quality and cryopreservation efficiency.

We feel that the importance of the reported findings makes this work of interest for the readership of In vitro Cellular &Developmental Biology - Animal. The present manuscript has not been published previously and is not being considered currently for publication in any other journal. All authors have approved the final article. The corresponding author is R.M. Pereira; Fax: +351 243 767 307; Phone: +351 243 767 380; INIAV Santarém, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal. Email: rosalnp@gmail.com. Finally, possible referees that are familiar with the methodologies used include the following:

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Looking forward to hear from you,

Best regards

RM Pereira

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