Chapter 5

Alterations in the expression of genes involved in lipid metabolism during bovine oocyte maturation and at the blastocyst stage in vivo vs. in vitro

O.A. Algriany[1], P.L.A.M. Vos[1], M.A. Sirard[2], S.J. Dieleman[1]

[1]Department of Farm Animal Health, Section Reproduction, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2]Centre de Recherche en Biologie de la Reproduction, Département des Sciences Animales, Universitéé Laval, Sainte Foy, Québec, Canada

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Abstract

Lipids, mainly long chain fatty acids, are in addition to their role as energy source implicated in many physiological processes such as precursors for the biosynthesis of membrane lipids, signaling transduction molecules and ligands for transcription factors. Fatty acids (FAs) and lipid transport, catabolism and synthesis require the correct temporal activity of several gene products. While the control of FAs transport and metabolism has been examined in different mammalian cells, regulation and coordination of lipid metabolism throughout maturation of mammalian oocytes and culture of early embryonic development is widely unknown. Therefore, transcript abundance of mRNAs involved in, 1) FAs transport, including FA translocase (FAT/CD36), FA transport proteins 1 (FATP1), 2) FAs β-oxidation like AMP-activated protein kinase (AMPK), carnitine palmitoyltransferase 1 (CPT-1), peroxisome proliferator-activated receptors αα (PPARα) and acetyl CoA carboxylase β (ACCβ), and 3) *de novo* FA synthesis including acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), were analyzed throughout oocyte maturation (2 h pre LH, 6 and 22 h after LH) and at the blastocyst stage (day 7) in vivo, using quantitative real time PCR. Presumptive competent oocytes were selected after oFSH treatment on the basis of the steroid profile in the enclosing follicle. Moreover, to determine the role of these transcripts in developmental competence, mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows, and to that of cultured oocytes from slaughterhouse ovaries at corresponding times of maturation and in vitro derived blastocysts. Taken together, our results showed an increase in mRNA implicated in FAs transport (CD36 and FATP1) and catabolism (AMPK, CPT-1) during oocyte maturation compared with the blastocysts, where there is a marked decrease or lack of these transcripts. In contrast, a sharp and significant increase was observed in mRNAs involved in de novo FA synthesis (FAS and ACCα) at the blastocyst stage compared to the oocytes. Moreover, our results showed that different oocyte microenvironments can have a profound effect on mRNA encoding components of FA utilization, catabolism and synthesis. In vitro produced blastocysts showed a significant decrease in ACCα compared to the in vivo derived ones. Our results suggest that an aberrant expression of these transcripts might contribute to oocytes and blastocyst developmental competence.

***Key words*:** gene expression; lipid metabolism; CD36; FATP1; CPT-1; ACCα

Introduction

Lipids, as well as carbohydrates and amino acids, are the three basic materials providing an important source of energy for all mammalian cells. Beside their crucial role as energy source, lipids, mainly long chain fatty acids (LCFAs) are precursors for the biosynthesis of membrane lipids, lipid signaling molecules and ligands for transcription factors that control metabolic activity. Depending on the cell type, and the energy needs, LCFAs can be imported into the cell from exogenous source, or generated endogenously from de novo synthesis, hydrolysis of triglycerides. Although, energy contained in lipids exceeds that in carbohydrates and proteins, much research relevant to energy needs during mammalian oocyte maturation and early embryonic development in vitro has been directed towards carbohydrates and amino acids and we know much less about the possible regulatory roles of lipids during mammalian oocyte maturation and early embryonic development.

Structurally, lipid content in bovine oocytes tends to increase progressively with oocyte growth [1], In addition, ranking the oocytes based on the commonly used indices for COCs quality [2] demonstrated a difference in fatty acids (FAs) composition and indicates that the appearance of the cytoplasm may reflect its lipid, FAs content and developmental competence [3, 4]. Biochemically, higher lipase activity [5] and a decrease in triglyceride content recorded during in vitro maturation of bovine oocytes [4] indicate that lipid may act as energy source during bovine oocyte maturation.

Oocyte and embryo FAs profiles and their rate of uptake have been proposed as a tool to predict oocytes and embryo quality. Haggarty et al [6] showed a reduction in the concentrations of linoleic and oleic acid in human embryos that failed to develop beyond 4-cell stage compared to that developed normally. Similarly, Zeron et al [7] found evidence that the seasonal changes in the bovine oocyte developmental competence might arise primarily due to changes in FAs profiles of ovarian follicular fluid and oocytes. Moreover, marked changes in linoleic acid levels of NEFA and phospholipids concentrations have also been reported between estradiol active and inactive bovine preovulatory follicles [8]. On the other hand, lipid accumulation during in vitro culture of early bovine embryos in medium containing FCS compared to serum free medium remains a source of debate for a number of studies. Earlier studies had indicated that culture in the presence of serum results in intracellular lipid accumulation and greater sensitivity to freezing [9-11] but more recent work suggests no difference in the post-cryopreservation survival [12]. Regardless of the presence or absence of serum, it is clear that culture of bovine embryos in vitro is

associated with a greater sensitivity to freezing during cryopreservation comparing to those matured in vivo [13]. Despite this evidence, the necessity of FAs and the pathways involved in regulation and coordination of lipid metabolism at the molecular level throughout maturation of oocytes and culture of early embryonic development remains largely unknown.

Examining some of the key genes controlling the major pathways of 1) FAs transport like FA translocase (FAT/CD36), FA transport proteins 1 (FATP1), and 2) FAs β-oxidation like AMP-activated protein kinase (AMPK), carnitine palmitoyltransferase 1 (CPT-1), peroxisome proliferator-activated receptors α (PPARα) and acetyl CoA carboxylase β (ACCβ), and 3) *de novo* FA synthesis including acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), may elucidate the possible role of FAs during oocyte maturation and early embryonic development, and the tasks played by these genes with regard to the control of lipid metabolism. Although the Free FAs can be taken up by diffusion through the plasma membrane [14], different proteins integrally associated with the plasma membrane that have been identified [15] can facilitate uptake and coordinate the import of Free FA in harmony with metabolic demands. The best characterized, including FAT/CD36 [16] and FATPs [17], changes in LCFA uptake have been observed to occur in parallel with changes in FAT/CD36 expression in rat heart and skeletal muscle [18], and a null mutation in FAT/CD36 reduced the uptake of FAs [19]. FATP1 is a well characterized member of a large family of plasma membrane proteins that increase FA import when expressed in cultured cells [17], disruption of the yeast *Saccharomyces cerevisiae* FATP1 has been shown to impair LCFA uptake growth [20].

Within most mammalian cells, controlling energy demands in the form of ATP are done through β oxidation of FAs in the mitochondria. Different genes have been involved in the transcriptional control of β oxidation. AMPK is an attractive potential candidate, and its role in this process has been suggested [21, 22]. AMPK is activated by low ATP/AMP ratios to serve as a fuel gauge for mammalian cells to protect against energy deprivation [23], its activation is associated with increased mitochondrial biogenesis [24] and enzyme content [25] in rat skeletal muscle. Activation of this enzyme in muscle increases FAs oxidation [26-28] and inhibits acetyl CoA carboxylase [25, 29]. Within the cytoplasm, FAs are activated to long-chain acyl-CoA by an acyl-CoA synthetase. The acyl part is then transferred into the mitochondria for oxidation by a complex of enzymes involving CPT-1 on the outer aspect of the inner mitochondrial membrane. Inside the mitochondrial matrix, long-chain acyl-CoA passes through the β-oxidation enzyme system to produce acetyl-CoA. It is generally accepted that in different mammalian cells β oxidation is regulated

through CPT 1 through different mechanisms, including changes in the activity and transcription rate of CPT 1, or its inhibitor malonyl-CoA the end product of ACCβ associated with mitochondrial membrane [30]. The transcription factor PPARα is a member of PPAR family that functions as a ligand-dependent transcription factor [31], its expression is more abundantly in tissues that are characterized by high rates of FAs oxidation (FAO), and is considered as the main subtype that mediates lipid-induced activation of FAO genes [32].

FAs are synthesized by a common biochemical pathway in all mammalian cells involving two key enzymatic reactions, the first is the carboxylation of acetyl-CoA in the cytosol to form malonyl CoA by ACCα [30], then FAS catalyzes the synthesis of LCFAs, by using acetyl-CoA as a primer, malonyl-CoA as a two-carbon donor for chain elongation, and NADPH for the reduction reactions [33].

While the control of fatty acid transport and metabolism has been examined in different mammalian cells, little is known about the oocyte control of fatty acid entry during the final stages of maturation. It is also well documented that oocyte quality obtained from gonadotropin-stimulated cows or matured in vitro can be variable due to many factors, among these factors which have been clearly demonstrated are the ATP content [34, 35] and lipid composition of the oocyte [7]. To adjust to changes in the demand of lipids during maturation and early embryonic development, oocytes and early embryos must precisely regulate and coordinate the expression of several genes. Therefore, this study investigates the expression of key mRNAs involved in lipid metabolism throughout bovine oocyte maturation and blastocysts stage in vivo. Additionally, we also analyzed the expression of these genes under in vivo and in vitro culture conditions to unravel part of the signals and mechanisms that participate in developmental competence.

Materials and Methods

Experimental design

At each time point of maturation, oocyte mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows and with that of cultured oocytes from slaughterhouse ovaries at corresponding times of in vitro maturation. Secondly, mRNA expression of the competent oocytes was compared with that

of in-vivo derived blastocysts. Finally, mRNA expression of day 7 in-vitro produced blastocysts was compared to in-vivo derived counterparts.

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [36] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation to determine changes in mRNA expression throughout maturation in vivo. Oocytes were selected on the basis of follicle size, the steroid profile in the enclosing follicle as shown in table 2 and, were assigned to replicates for QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. In vivo blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. In-vitro oocytes were produced after maturation in vitro (IVM) using oocytes obtained from an abattoir, while in-vitro produced blastocysts were collected after fertilization and culture in-vitro of the in-vitro matured oocytes till the blastocysts stage. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatment

Normally cyclic Holstein-Friesian cows (n=36) were selected and treated for superovulation using the protocol as described before [36] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [37] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [38]. Oocytes and follicular fluids were collected following OVX at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow by laparotomy through flank incision under local infiltration anesthesia [39]. Ovaries were collected in 0.9% (w/v) NaCl at 37^{o}C and immediately transported to the laboratory. The contents of each follicle >

9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after incubation for one minute with 100 µl 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, U.S.A.) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually in at -80°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were sub-divided on the basis of the concentration of steroids in the fluid of the enclosing follicle. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH, > 0.5 µmol/L 6 h after LH, and progesterone > 0.5 µmol/L 22 h after LH were considered to be competent (Table 2). Oocytes from follicles with unmistakably deviating steroid concentrations were assigned to the respective non-competent sub-groups, that is with estradiol < 0.37 µmol/L before and 6 h after LH, and with progesterone < 0.38 µmol/L 22 h after LH. The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4 ºC, plasma was stored at -25 ºC.

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [37] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

In vitro maturation (IVM) of immature oocytes

Bovine ovaries were collected from abattoirs, and transported in a thermos flask within 3 hours of collection. After washing with water and saline solution, cumulus oocyte complexes (COCs) were aspirated from follicles 3-6 mm in diameter, washed in HEPES-buffered TCM-199 (Gibco BRL, Paisley, UK), and selected on the basis of their morphology for in vitro maturation according to the density of their cumulus cell layers, and randomly allocated in groups of 50 COCs per well to a 4-well culture plate (Nunc A/S, Roskilde, Denmark). In vitro maturation of the COCs was performed in 500 µl TCM-199 per well, supplemented with 10% (v/v) fetal calf serum, 4 µg FSH/ml, and 6 µg LH/ml (Sioux Biochemical Inc., Iowa, U.S.A.), and 0.1 mM cysteamine (Sigma Chemical Co., St Louis, MO, U.S.A.) for 22 h at 38.5ºC under an atmosphere of 5% CO2 in air with maximum humidity. At the beginning (0 h), 6 h and 22 h of the maturation, cumulus cells of 13 oocytes from three different batches were denuded by vortexing and stored at -80 ºC.

In vitro embryo production (IVP)

Procedures for in vitro maturation were performed as described previously, after maturation, oocytes in COCs were fertilized in vitro according to the procedure described by Parrish et al. [40] with minor modifications [41] using frozen-thawed semen from a bull of proven fertility. The presumptive zygotes were freed from cumulus cells 20 h after IVF by vortexing, and a maximum of ten zygotes was placed in a 20 µl droplet of synthetic oviductal fluid (SOF) medium [42] supplemented with essential and non-essential amino acids (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.1% (w/v) BSA (Sigma-Aldrich), under oil (Reproline medical GmBH, Rheinbach, Germany) and cultured at 39°C, in humidified air containing 5% CO_2 and 7% O_2. On day 4 after IVF the number of cells per cleaved embryo was scored and all cleaved embryos were transferred to fresh SOF droplets. The developmental stage of the embryos was assessed at day 7 after IVF. Four groups of 5 expanded blastocysts were rinsed in PBS and stored at -80°C until RNA extraction.

Collection of in-vivo derived blastocysts

Superovulation was induced using an eCG/monoclonal anti-eCG/PG treatment scheme [37]. Cows were inseminated with one straw into each uterine horn, 10 h after the LH peak. Seven days later the embryos were non-surgically recovered, embryonic developmental stage and general morphological appearance were assessed by stereo microscopy, once

qualified to those in morphological grades I and II [43], frozen for storage in liquid nitrogen until use.

RNA isolation, precipitation

Total RNA was prepared from at least three replicates at each maturation stage, except 22 h normal, 6 and 22 h deviant oocytes, where 20, 18 and 11 oocytes were collected, respectively. Each replicate containing from 9 to 13 pooled oocytes. Four replicates of day 7 blastocysts were used each containing 5 blastocysts, the RNA was then isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit, Stratagene, San Diego, CA, U.S.A.). The RNA was recovered by two subsequent 50-µl elutions with warmed (60°C) elution buffer provided in the kit.
RNA was then precipitated with 250 µl of 100% ethanol (EtOH) and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX, U.S.A.) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000 g. The pellet was then washed with 75% EtOH and resuspended in 15 µl of water for real-time PCR analysis.

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25 °C, 30 min at 42°C, and 5 min at 85°C.
Primer sets were designed by using Beacon Designer 4 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.), from bovine sequences from NCBI, the primers used in the study are shown in table 1. The specificity of the primers was confirmed by sequencing and confirmation of the PCR product size on a standard 2% agarose gel with ethidiumbromide (EtBr). The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantification with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, the Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as determined using oocytes from slaughter house ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from at least three different groups of pooled oocytes, except 22 h normal, 6 and 22 h deviant oocytes, where 2, 2 and 1 group were used, respectively (see table 2).

Table 1. Information on the primers used for real-time PCR

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (^{o}C)
CD36	NM_174010	F 5'-tcattgctggtgctgtcattgg-3' R 5'-aactgtcacttcatctggattctgc-3'	209	59
FATP1	NM_001033625	F 5'-caagagcctggtcaagttc-3' R 5'-cgtgtagatgtagaagagtcg-3'	157	57
AMPAKγ1	BT025456	F 5'-aagatagaaacttggagagaggtg-3' R 5'-aacttgaggaacttgaggatgc-3'	197	59
CPT-I	NM_001034349	F 5'-ggtcaacagcaactactacg-3' R 5'-tgaacatcctctccatctgg-3'	188	52
PPARα	NM_001034036	F 5'-tcgtttcctcctttaccttc-3' R 5'-tctgtgtccaccatctcc-3'	170	54
ACCβ	AJ966324	F 5'-tacctgcgggcgtctgag-3' R 5'-gctgattctggtgtagtcttgg-3'	192	56
ACCα	NM_174224	F 5'-aagcaatggatgaaccttcttc-3' R 5'-gatgcccaagtcagagagc-3'	197	58
FAS	NM_001012669	F 5'-ctctccctcagccgttcg-3' R 5'-gcctgtcatcatctgtcacc-3'	168	63
GAPDH	BTU85042	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

Bos taurus CD36, Fatty acid translocase; FATP1, *Bos taurus* fatty acid transport protein 1; AMPAKγ1, *Bos taurus* AMP-activated protein kinase gamma 1; CPT-1, *Bos taurus* carnitine palmitoyltransferase 1; PPARαα, *Bos taurus* peroxisome proliferator-activated receptors α; ACCβ, *Bos taurus* acetyl-CoA carboxylase β; ACCα, *Bos taurus* acetyl-CoA carboxylase α; FAS, *Bos taurus* fatty acid synthase; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase

Samples and standard curves were amplified on the same run with the same PCR master mix.

The thermal cycling program starts with an initial denaturation step at 95°C for 3 min, and is followed by 45 PCR cycles (dissociation for 5 sec at 95°C, annealing for 5 sec at temperature showed in table 1, and elongation for 20 sec at 72°C), one melting cycle consisting of 5 sec at 95°C, 30 sec at 72°C, and a step cycle up to 95°C (0.3°C/sec transition rate), and finally a cooling down cycle at 40°C. Amplification of the GAPDH mRNA [44, 45] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to confirm amplification. Product sizes and annealing temperatures for each gene are presented in table 1.

Statistical analysis

Data are presented as the mean ± SEM. Initially, expression analysis data of the oocytes from follicles with a normal profile were subjected to one-way ANOVA and Bonferroni test was used as a post hoc for comparison of individual means to assess the effect of maturation stages (-2, 6, 22). Secondly, data from oocytes classified normal in the prematuration stages (-2) were compared using un-paired Student's t-test to the in vivo derived blastocysts to study the difference in gene expression between oocytes and blastocysts. Thirdly, differences within each oocyte time group (follicles with a normal profile, deviant profile and oocytes matured in vitro) were analyzed using one-way ANOVA. Difference between in vivo and in vitro derived blastocysts was analyzed using Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

In total 171/241 before LH, 135/183 at 6 h after LH and 60 oocytes/ 93 follicles were retrieved resulting in 71, 74 and 65% recovery, respectively. The number of oocytes classified as competent and non-competent based on steroid and follicle size are presented in table 2.

Table 2.: Steroid concentrations in follicles with competent and non-competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge

	notes	2 h before LH (Pre)	6 h after LH (Post)	22 h after LH (Post)
Normal follicles	[1]			
- number		47	50	20
- estradiol 17β	[2]	1.64 ± 0.12	0.84 ± 0.04	0.11 ± 0.01
- progesterone		0.15 ± 0.01	0.51 ± 0.01	0.75 ± 0.05
- follicle diameter	[3]	13 ± 0.4	13.5 ± 0.3	13 ± 0.5
- number of cows	[4]	13	9	8
Deviant follicles				
- number		52	18	11
- estradiol 17β		0.21 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
- progesterone		0.09 ± 0.004	0.58 ± 0.06	0.16 ± 0.03
- follicle diameter		12 ± 0.3	12 ± 0.4	12 ± 0.8
- number of cows		7	7	3

[1] competent: oocytes from functional follicles on the basis of steroid profile
[2] steroid concentrations ± SEM in μmol/L follicular fluid
[3] mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used
[4] number of cows from which oocytes were retrieved

Results of gene expression were used to organize group of genes according to their function.

Group 1. Fatty acid transport: within the oocytes selected from normal follicles, levels of CD36 (fig. 1, A) and FATP1 (fig. 1, B) mRNA were relatively high before, 6 and 22 h after maturation, but no significant ($P < 0.05$) differences were observed between the different time groups (from normal follicles). The expression pattern of this group at the blastocysts stage characterized by the absence (CD36) or low levels of mRNA (FATP1). Within each time group, there were marked changes in the expression between the groups but it was significant only between oocytes collected from FSH stimulated cows classified as normal and deviant in the pre-maturation groups for both mRNA.

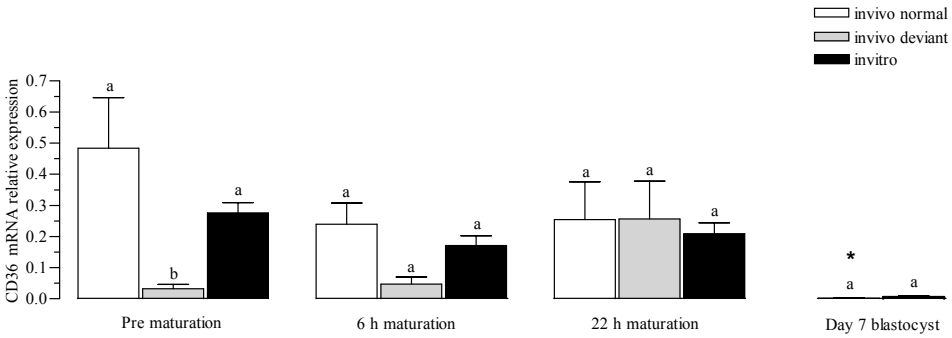
Group 2. Fatty acid β-oxidation: mRNA levels varied across this group, with the level of AMPK (Fig.1, C) and CPT-I (fig. 1, D) high during the pre-maturation and 6, 22 h after maturation but there were no statistical significant differences between the time groups ($P < 0.05$). A marked reduction in the mRNA expression for both mRNA at the blastocyst stage,

CPT-1 mRNA was not detected in in vivo derived blastocysts. The gene expression of AMPK and CPT-1 was significantly ($P < 0.05$) reduced in the oocyte scored as bad during pre-maturation compared to the good one, CPT-1 expression in the oocyte matured in vitro was significantly altered compared to the oocytes classified as normal, whereas both transcripts remained unchanged during 6 h of maturation. A significant difference within the 22 h group in the expression of AMPK, between in vivo normal, deviant, and those matured in vitro was observed.

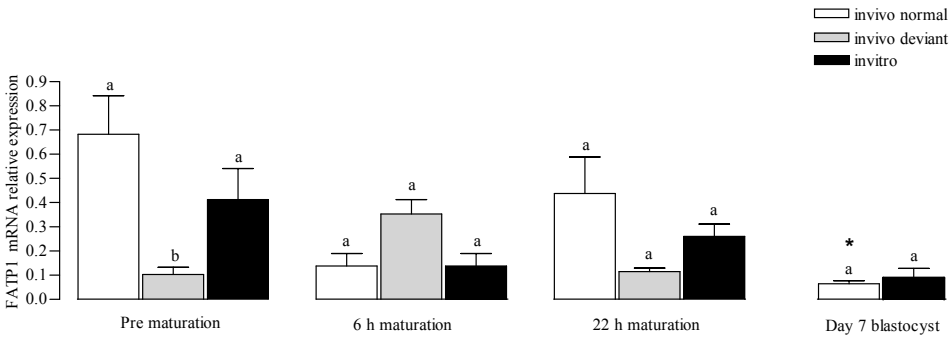
The levels of PPARα (Fig. 1, E) mRNA remained unchanged between the different time groups and also between oocytes and blastocysts. A significant difference between the oocytes collected from follicles with normal profiles was seen compared to those scored as deviant and matured in vitro ($P < 0.05$). Whereas no significant alterations in PPARα mRNA expression between in vivo and in vitro blastocysts were detected. Similarly, ACCβ (Fig. 1, F) mRNA levels were not significantly different ($P < 0.05$) within the oocyte time groups (-2, 6, 22) and also between the oocytes and the blastocysts.

Group 3. Fatty acid synthesis: A similar pattern of changes characterize both member of this group, ACCα (Fig. 1, G) and FAS (Fig. 1, H). No differences between different time groups during oocyte maturation and a sharp and significant increase in both mRNA levels during the blastocysts stages compared to oocyte maturation. A stastically significant difference ($P < 0.05$) was observed in the expression of ACCα between in vivo derived blastoysts and those cultured in vitro.

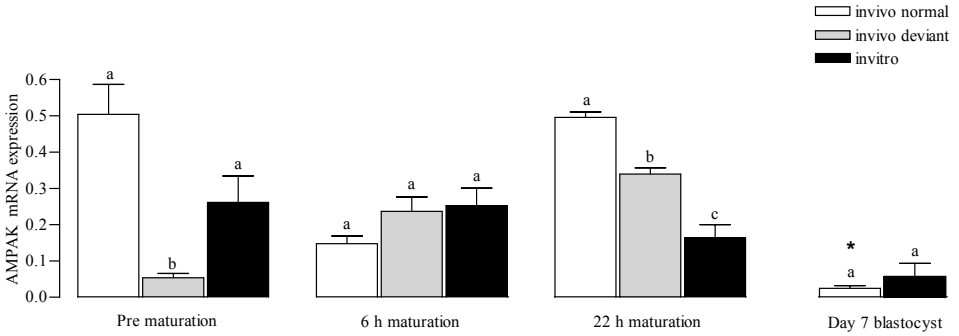
Figure 1. Quantification of mRNA levels by real-time PCR of genes involved in lipid metabolism (A to H) in bovine oocytes from cows undergoing superovulation with FSH scored as normal or deviant based on steroid profile and follicle sizes and oocytes collected from slaughterhouse ovaries and matured in vitro.
a, b, c: significantly different within the time groups (in vivo normal, in vivo deviant and in vitro oocytes) ($P < 0.05$).
* Significantly different from the oocytes



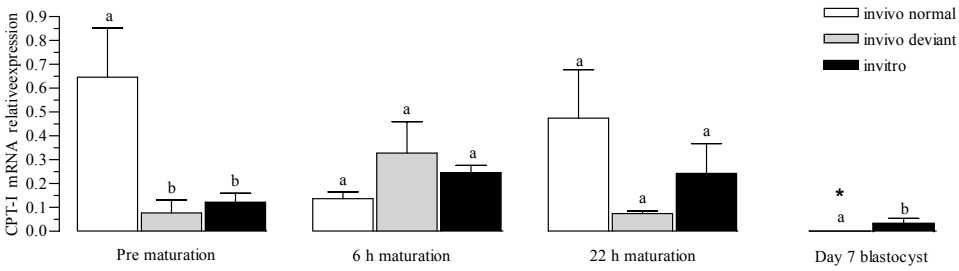
A. Fatty acid translocase/CD36 mRNA



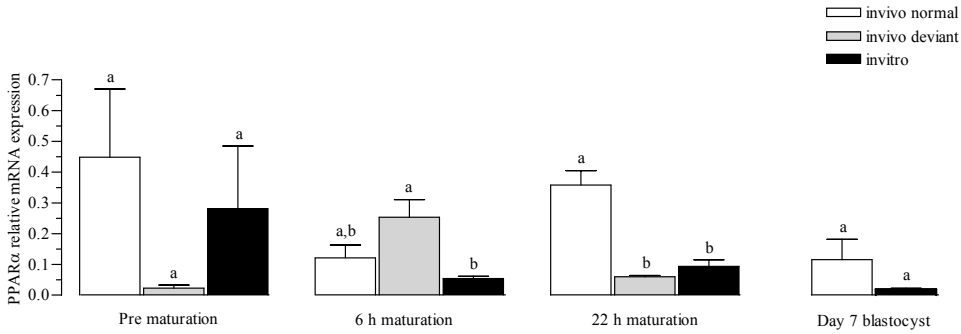
B. Fatty acid transport protein1



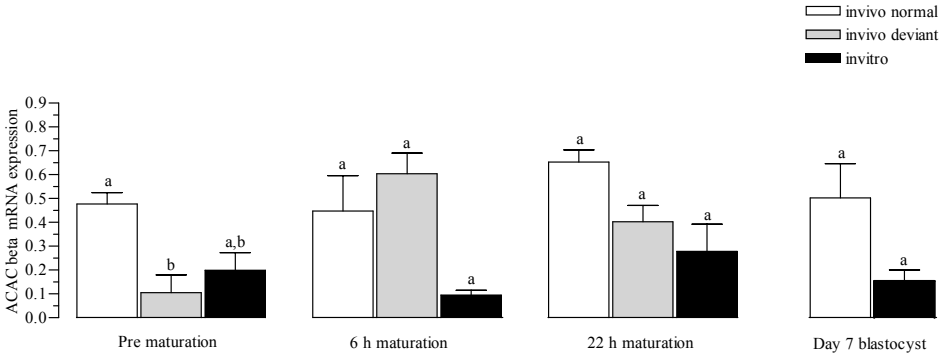
C. AMP-activated protein kinase γ1



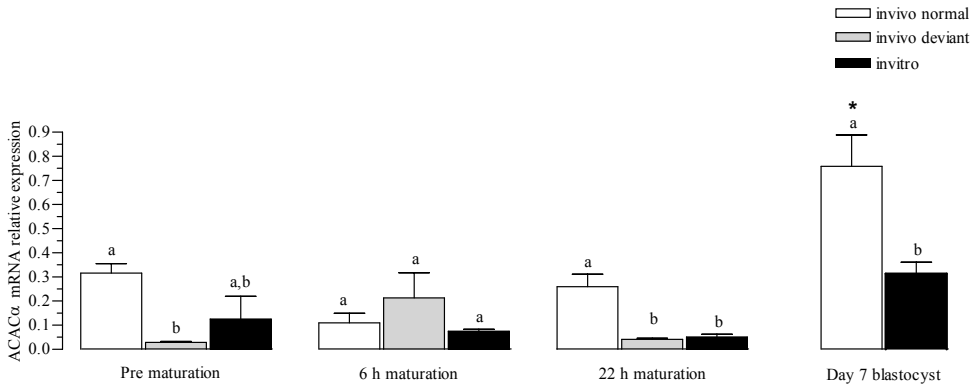
D. Carnitine palmitoyltransferase I



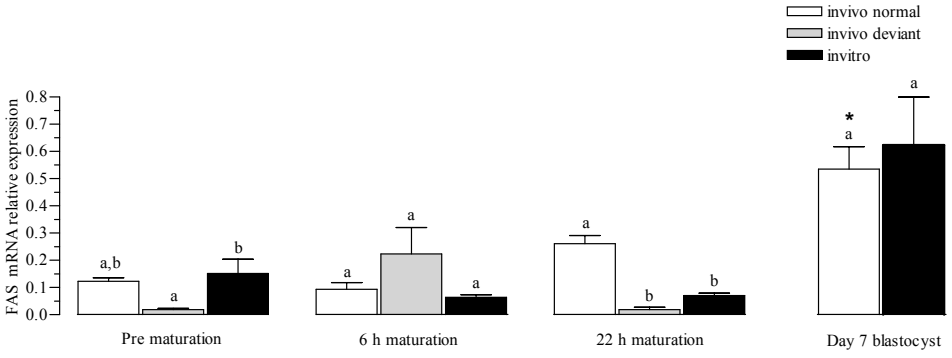
E. Peroxisome proliferative activated receptor alpha



F. Acetyl CoA carboxylase beta



G. Acetyl CoA carboxylase Alpha



H. Fatty acid synthase

Discussion

Our data suggest that, under physiological conditions, 1) during oocyte maturation, exogenous FAs supply is probably the preferential source of LCFA and energy source, based on the higher expression of transcripts involved in FAs transport and key regulatory enzymes involved in β-oxidation of FAs. 2) At the blastocysts stage, the parallel increase in the mRNA abundance of FAS and ACCα suggests an increased rate of lipogenesis which may be needed to support earlier embryogenesis. 3) A lower reliance on FAs as a source of energy at the blastocysts stage as shown by a lack or sharp decrease of mRNAs implicated in FAs catabolism. 4) Exogenous FSH stimulation and in-vitro culture conditions can results in extreme deviations in several transcripts involved in lipid metabolism during oocyte maturation and blastocysts formation.

CD36 expression favors tissues with high metabolic capacity for LCFA, such as adipose, heart and muscle, whereas it is absent from tissues like brain that do not utilize LCFA [16], its mRNA expression is induced by LCFA and not by short chain FAs [46]. The CD36 mRNA has been reported previously in mouse oocytes using the microarray technique [47]. The mechanisms by which oocyte competence is affected by FAs are not completely understood. However, the higher abundance of plasma membrane fatty acid transport mRNAs (CD36 and FATP1) suggests that the effect of LCFAs on oocyte maturation is direct and not mediated by cumulus cells. It also may indicate that the oocyte can not synthesize certain LCFA and that exogenous FAs are needed to support certain oocyte requirements. It is well known that animals can synthesize most fatty acids, with the notable exceptions of polyunsaturated fatty acids (PUFA) like linoleic and linolenic acids, since animals must have a dietary source of these FAs and therefore they are considered as essential FAs. The essential FAs have different important functions, the most important is structural contribution to the maintenance and function of the cellular membranes [48]. Alterations in FAs composition and lipid contents during oocyte growth are a well documented phenomenon. Zeron et al [7] reported that the decline in the developmental competence of oocytes Holstein cows was mainly associated with a significantly lower linoleic acid content in the oocyte membrane. The same author reported changes in the ewes oocyte's FAs profile by PUFA diet supplementation which associated with better oocyte quality and chilling resistance [49]. As a result, it appears likely that the reduction in the synthesis of mRNA for the genes encoding CD36 and FATP1 observed in oocytes classified as deviant and oocytes matured in vitro may reflect later reduced LCFAs import into the oocyte that may affect fundamental cellular processes such as FA incorporation into triglycerides, membrane components, signal transduction pathways, gene expression

and FAs generated energy. Moreover, the co-expression of both CD36 and FATP1 in the oocyte may indicate that they have a different function since a number of studies using 293 cells [50] and FATP1 knock-out mice have reported that LCFAs taken by FATP1 were channeled into triglycerides synthesis. This synthesis exceeded oxidation in the mitochondria [51], whereas FA/CD36 was identified recently on mitochondria isolated from rat and human skeletal muscle and found to be involved in LCFA oxidation [52, 53].

Little is known about the nutritional requirement of the bovine oocyte. Using FCS as IVM medium supplement can promote oocyte developmental competence. Its efficacy has been ascribed to its superior protein quality, amino acids, vitamins, heavy-metal chelators and unknown growth factors, making it the most effective IVM component. Oocyte triglyceride and the total lipid content have been shown to increase in oocytes matured in vitro in a medium containing FCS compared to those matured in serum free medium [4]. This may strongly indicate indirectly that the beneficial effect of FCS as a major component of bovine oocyte IVM medium may be also due to FAs contents. It has been suggested that decrease or increase in the expression of different lipid metabolic genes are modulated by direct contact with FA, that alter chromatin structure and transcription through direct effect on histone deacetylase activity, for instance, FATPs, CPT-1, PPAR has been shown to be upregulated by FAs whereas FAS, ACC are down-regulated (for review see [54]. Based on the above, decreased FA oxidation may reflect a defect in fatty acid oxidation genes activation due to alterations in CD36 and FATP1 abundance as a result of poor culture conditions.

AMPK activity depends on the AMP/ATP ratio that is changed during oocyte maturation. Cyclic AMP degradation results in the accumulation of AMP which binds and activate AMPK directly by binding to its γ subunit, mechanisms other than changes in the cellular AMP-to-ATP ratio have also been reported to activate AMPK [55]. However, our results showed a higher expression of AMPK mRNA even in the oocytes collected before LH surge and maturation in vitro, which is consistent with the recent finding using mouse oocytes [56]. AMPK is a stress-activated enzyme, and even gentle physical manipulation can stimulate the kinase [57, 58]. In mouse oocytes, AMPK activation has been shown to precede GVBD and to play an important role in meiotic resumption [56]. The simultaneous increase of AMPK, CD36, FATP1, and CPT-1 suggests that AMPK is associated with FAs catabolism as shown previously in most mammalian cells.
Cyclic AMP-dependent protein kinase (PKA) and AMPK phosphorylates and inactivates ACC, decreases malonyl-CoA levels and increases fatty acid oxidation both in vivo and in vitro [59, 60]. This phosphorylation regulation may be also an important regulatory aspect

of fatty acid metabolism during oocyte growth and maturation. Higher cAMP within the oocytes activates PKA before maturation and maintain meiotic arrest while a decrease in intra-oocyte cAMP initiate oocyte maturation [61]. The expression of both ACC and AMPK may suggest that the oocyte uses the same mechanism to phosphorylate and inactivate ACC, which stimulates fatty acid oxidation by different pathways as for other somatic cells through stimulation of PKA following elevation of cAMP levels. Thus, cAMP signaling is required not only for meiotic arrest and resumption but also for their intimate coordination of the several oocyte metabolic processes.

CPT-1 resides on the outer mitochondrial membrane and catalyses the conversion of palmitoyl-CoA to palmitoylcarnitine, which is the rate limiting step in the transfer of long-chain fatty-acylCoAs from the cytosol to the mitochondria for oxidation [30, 62]. Its activity is inhibited by malonyl-CoA, therefore, a decrease in the malonyl-CoA removes inhibition of CPT-1 and allows fatty acid oxidation to increase to meet the increased energy requirement. In mammals, two different CPT-1 enzymes are encoded by two different genes, liver CPT-1 and muscle CPT-1 [63]. Muscle CPT1 has a crucial role in controlling the rate of β-oxidation in heart and skeletal muscle and is more sensitive to malonyl-CoA inhibition [64]. Therefore, we decided to specifically amplify the muscle isoform. In the present study, we found that a general increase of CPT-1 mRNA levels takes place during early oocyte maturation or results from the polyadenylation of stored pools and lack of expression at the blastocysts stage. These findings together with the results published recently, using methyl palmoxirate (MP), the inhibitor of CPT-1 enzyme activity [65], indicate an increase in the rate of β-oxidation and important role of fatty acids as a source of energy during oocyte maturation. The lower expression of CPT-1 during prematuration in the oocytes matured in vitro and oocytes from deviant follicles may indicate a dysregulation of fatty acid oxidation activity causing a shortage of ATP and hence lower oocyte developmental competence [34]. These results are not surprising, steroid hormones have a significant control on cellular lipid in metabolically active tissues [66]. In skeletal muscle, ovariectomy suppresses the maximal activity of CPT-1 and the activity of this enzyme is up-regulated following treatment with E2. Moreover, In aromatase deficient mice (ArKO), exogenous E2 is necessary to maintain the gene expression and enzyme activity of the hepatic β-oxidation pathway [67] suggesting a capacity of E2 to regulate the expression of genes implicated in lipid metabolism. Low mitochondrial β-oxidation could also change the fatty acid composition within the oocyte since a marked change has been reported in the linoleic acid levels occuring between E2 active and inactive bovine preovulatory follicles [8]. In addition to its role in β-oxidation, lower CPT-1 activity has also been reported to enhance palmitic acid (PA) induced cell death and PA anti-

proliferative effect [68]. Possibly, this is also the case in the oocytes from deviant follicles and in those matured in vitro, the decrease in CD36 and FATP1 may result in the preferent use of FAs other than LCFA through passive diffusion, and lower CPT-1 mRNA expression may decrease the utilization of LCFA. This altered mRNA expression may affect the balance in the lipid FAs composition ratios at the physiological level. Thus, understanding of the import of FAs, the specific metabolic fates of different FAs and the manner in which these are regulated may contribute to the better design of oocyte IVM medium.

At the blastocysts stage, we found a small increase of a CPT-1 mRNA in the ones cultured in vitro compared to the in vivo counterparts. This may contribute to incomplete blockage of β-oxidation pathway in blastocyst cultured in vitro. It has been hypothesized that metabolically active blastocysts have less chance to develop to term after transfer and the metabolically inactive are more likely to develop after transfer [69, 70]. This slight increase in CPT-1 mRNA in the blastocysts cultured in vitro may be also related to the lower levels of ACCβ mRNA expression in in vitro blastocysts that probably was not enough to block CPT-1 activity. The block of CPT-1 activity probably induces more acetyl-CoA to go to lipid synthesis instead of β-oxidation. Our finding that the oocytes express CPT-1 mRNA before and during maturation and the lack of the expression at the blastocysts stage, support and explain recent findings that blocking of β-oxidation using CPT-1 inhibitor methyl palmoxirate before or during maturation of bovine oocytes significantly decreases oxygen consumption, while no difference was reported in the oxygen consumption of day 7 blastocysts [65]. Furthermore, based on the lack of CPT-1 mRNA expression in blastocysts, it appears that the lipids synthesized during early embryonic development were not used as a source of energy, and may be directed for membrane structure and organogenesis. Therefore, energy requirements at this stage are preferentially supplied by carbohydrates and amino acids as shown previously [71, 72]

The potential role of PPARα in regulating fatty acid oxidation has been well demonstrated. Activation of PPARα results in increase in the gene expression of many FAO enzymes [73, 74]. In the present study, the synergistically up-regulation of fatty acid transport and oxidation genes and PPARα mRNA may indicate that these genes are targets for the activation by PPARα. Fatty acids especially highly unsaturated fatty acids have profound effects on gene expression, acting on the genome through PPAR leading to changes in metabolism, growth and cell differentiation [75, 76]. Fats rich in PUFA like fish oil increase activity and mRNA levels of hepatic FAO enzymes [77]. Polyunsaturated fatty acids have also been considered as a natural ligands or activators of PPAR [78]. A study

using PPARα-deficient mice suggested that PUFA (fish oil) up-regulates FAO enzyme gene expression through a PPAR dependent mechanism [79]. In the same experiment, it has been shown that PUFA-mediated suppression of lipogenic gene expression does not require PPARα. Therefore, the lack of certain FAs may explain the difference in the abundance between oocytes from normal or deviant and those matured in vitro.

In the present study, the expression of both isoforms of ACC was identified in the oocyte and the blastocyst stage. The malonyl-CoA, the product of ACCα and ACCβ, exists in two separate compartments in the cell, cytosol and mitochondria, respectively [80] and do not mix. The cytosolic malonyl-CoA is used in FAs synthesis, while the mitochondrial part regulates CPT-1 and hence, FAs oxidation. ACCα is the dominant isoform and contributes to most of the malonyl-CoA. A study using ACCα knockout mice reported an essential role in embryo development [80] whereas ACCβ works as a regulator of FAs oxidation [81]. Oocytes from deviant follicles and those matured in vitro, showed a lower mRNA level of ACCα at the GV and MII than those classified as normal. Since ACCα is involved in fatty acid elongation, this indicated that the activity of ACCα is not completely blocked and certain enzyme activity is needed. A *Saccharomyces cerevisiae* mutant of ACCα grown at restrictive temperature developed an altered nuclear envelope and showed severe abnormalities in spindle formation to become arrested in the G2-M phase of the cell cycle loosing viability [82]. In addition, our study showed a statistical significant decrease in ACCα mRNA abundance in the blastocysts obtained using IVP system. This may indicate a change in the quantity of synthesized lipid, resulting in a lack of certain LCFAs needed for membrane integrity and structure. In yeast, inactivation of ACCα completely inhibits growth and cause cell death even after supplementation of FAs [83]. Moreover, inhibition of ACCα in a prostate tumor cell line has been shown to induce a growth arrest and apoptosis [84]. LCFAs and the degree of unsaturation of membrane lipids are the major factors determining the structure and fluidity of lipid membranes and hence chilling sensitivity [85, 86]. Thus, membranes with unsaturated acyl chains in phospholipids remain fluid at lower temperatures than do membranes with saturated lipids [87, 88]. In view of the high degree of conservation of ACCα in most living organisms, a similar function of this gene is possible in the oocytes and early embryos. Collectively, these findings may explain partly the lower developmental competence of the in-vitro matured oocytes and those from deviant follicles with FSH stimulated cows. They probably explain the sensitivity of IVP blastocysts to chilling compared to the in-vivo derived ones, and represent strong candidate markers for developmental competence of oocytes and blastocysts.

FAS, catalyses the *de novo* synthesis of saturated FAs such as, myristate, palmitate and stearate using acetyl- and malonyl-CoA. Targeted deletion of the FAS gene results in mice that die in utero [89], and more than 50% of heterozygous FAS knockout mice fail to survive embryonic development [89]. Expression of FAS is controlled primarily at the level of transcription and in response to both hormonal and nutritional signals [90, 91]. In the present study, contrary to our anticipations, there was a marked increase of FAS mRNA abundance at the blastocysts stage compared to that in oocytes suggesting an important role in the FAs or triglycerides synthesis. However, the absence of difference in the abundance of FAS mRNA between the in vivo derived blastocysts and those obtained from in vitro, may suggest a less critical role in the developmental competence of early embryos.

Changes in transcription cannot be equated to changes in functional pathways because transcripts have to be translated, and proteins may require post-translation modification in order to become functional, [92]. Minimal polyadenylation is required for reverse transcription but it does not ensure translation. To validate translation, protein levels have to be measured and often they increase as the RNA level drops before the maternal-zygote transition (MZT) in bovine while it is the opposite after the MZT [93]. Therefore, changes in mRNA levels needed the support of functional biochemical assays. Unfortunately, current protein and enzymatic activity evaluation techniques will require a large quantity of oocytes, which are very difficult to obtain and limit such assays in the case of oocytes from in vivo material. Identification of mRNA expression will provide the basis to define the complex regulation of lipid metabolism.

In conclusion, the increase in mRNA levels implicated in FAs transport and catabolism during oocyte maturation compared to that during early embryonic development is probably due to an increased metabolic activity resulting from higher energy requirements. Moreover, we have shown that different oocyte and blastocyst microenvironment and culture conditions can have a profound effect on mRNA encoding components of FA utilization and synthesis. We propose that the resultant changes in the oocyte and blastocyst gene expression in vivo vs. in vitro contribute to the observed decrease in the developmental competence of embryos by alteration in lipid metabolism.

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