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## Review article

## Lipidome signatures in early bovine embryo development



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## A B S T R A C T

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Mammalian preimplantation embryonic development is a complex, conserved, and well-orchestrated process involving dynamic molecular and structural changes. Understanding membrane lipid profile fluctuation during this crucial period is fundamental to address mechanisms governing embryogenesis. Therefore, the aim of the present work was to perform a comprehensive assessment of stage-specific lipid profiles during early bovine embryonic development and associate with the mRNA abundance of lipid metabolism-related genes (*ACSL3*, *ELOVL5*, and *ELOVL6*) and with the amount of cytoplasmic lipid droplets. Immature oocytes were recovered from slaughterhouse-derived ovaries, two-cell embryos, and eight- to 16-cell embryos, morula, and blastocysts that were *in vitro* produced under different environmental conditions. Lipid droplets content and mRNA transcript levels for *ACSL3*, *ELOVL5*, and *ELOVL6*, monitored by lipid staining and quantitative polymerase chain reaction, respectively, increased at morula followed by a decrease at blastocyst stage. Relative mRNA abundance changes of *ACSL3* were closely related to cytoplasmic lipid droplet accumulation. Characteristic dynamic changes of phospholipid profiles were observed during early embryo development and related to unsaturation level, acyl chain length, and class composition. *ELOVL5* and *ELOVL6* mRNA levels were suggestive of over-expression of membrane phospholipids containing elongated fatty acids with 16, 18, and 20 carbons. In addition, putative biomarkers of key events of embryogenesis, embryo lipid accumulation, and elongation were identified. This study provides a comprehensive description of stage-specific lipidome signatures and proposes a mechanism to explain its potential relationship with the fluctuation of both cytoplasmic lipid droplets content and mRNA levels of lipid metabolism-related genes during early bovine embryo development.

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## 1. Introduction

Mammalian preimplantation embryonic development is a complex, conserved, and well-orchestrated process

involving dynamic molecular and structural changes during the embryogenesis period [1]. Embryos must undergo important events, such as fertilization, cleavage, epigenetic reprogramming, compaction, differentiation, and blastulation, for the proper development and pregnancy establishment [2,3]. A series of holistic experiments evaluating transcriptome and proteome during early bovine embryonic development have been performed

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addressing the fluctuation of gene transcripts and proteins associated with important biologic process that occurs during this period [1,3–5].

Previous studies were conducted to assess the lipidome of mouse [6] and bovine [7,8] oocytes and preimplantation embryos. However, a comprehensive lipid analysis of stage-specific lipidome signatures is still lacking.

Lipids are essential biomolecules of cells as a component of the plasma membrane and the membranes of various organelles. They are also directly involved in signal transduction as lipid mediators including phosphatidylinositols, sphingolipids, and eicosanoids [9]. Lipids also play important roles in a series of key biologic process including cell proliferation, migration, differentiation, chemotaxis, pinocytosis, survival, and metabolic changes [10,11]. Lipids potentially serve as signaling molecules that help co-ordinate fundamental events during embryo development, implantation, and post-implantation growth [9,12,13].

Triglycerides (TAG), the major lipid class found in the cytoplasm of mammalian cells, are stored as lipid droplets [14,15]. For the early embryonic development, lipid droplets seem to work as a source of energy for oocytes and embryos [16,17]. In eukaryotic cell membranes, however, phospholipids (PLs) are the most abundant lipids [18]. Phospholipids, most particularly, phosphatidylcholines (PC), phosphatidylethanolamine (PE), and sphingomyelins (SM) are structural units of functional membranes, and their composition determines most of the physicochemical cell membrane properties, including fluidity, permeability, and thermal phase behavior [19].

It has already been described the effect of different environmental conditions in the number of cytoplasmic lipid droplets [20] and lipid profile [21] of *in vitro*-produced bovine embryos. Additionally, changes in lipid membrane composition were monitored by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) comparing *Bos taurus indicus* and *Bos taurus taurus* and *in vivo*- and *in vitro*-derived blastocysts [22]. Furthermore, lipid-processing molecules (long-chain acyl-CoA synthetases [ACSLs] and elongation of very long fatty acids [ELOVLs]) that may be potentially involved with membrane lipid metabolic pathways in bovine embryos were identified [23].

ACSLs are a family of enzymes (ACSL1, 3, 4, 5, and 6) that activate long-chain fatty acids to generate long-chain acyl-CoA, essential substrate for the synthesis of various lipid species, including TAG, cholesterol ester, and PL [24,25]. These enzymes have already been related to the modulation of cell fatty acid uptake [26]. ELOVL family members (ELOVL1 to 7) are responsible for elongation of fatty acids to generate very long chain of saturated, monounsaturated, and polyunsaturated fatty acids [27,28]. Both ACSL and ELOVL enzyme family members are expected to be involved in the lipid metabolism of preimplantation bovine embryos. In a preliminary study, *in vitro*-produced embryos that presented greater lipid content than *in vivo* counterparts also reported increased mRNA levels for ACSL3, whereas *Bos taurus* embryos that had more lipid droplets and increased cryosurvival compared with *Bos indicus* embryos also presented with increased mRNA levels for ELOVL5 [23]. Additionally, ELOVL6 enzyme is known to

elongate palmitic and palmitoleic fatty acids [28], abundant fatty acids during early embryo development [29].

The objective of present work was, therefore, to comprehensively assess the stage-specific lipid profiles during early development of *in vitro*-produced bovine embryos and associate with the mRNA levels of lipid metabolism-related genes (ACSL3, ELOVL5, and ELOVL6) and with the amount of cytoplasmic lipid droplets.

## 2. Materials and methods

### 2.1. Reagents used

All materials were acquired from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA), except when specified.

### 2.2. Experimental design

To obtain the stage-specific lipid profiles, cytoplasmic lipid droplets content and mRNA transcript levels of lipid metabolism-related genes during early bovine embryo development, five *in vitro* developmental stages were assessed: immature oocytes (–24 hours post-insemination [hpi], considered as the start point of lipids measurement), two-cell embryos (32–40 hpi), eight- to 16-cell embryos (72 hpi), morulas (120 hpi), and blastocysts (168 hpi). Two different *in vitro* culture environmental conditions were used for embryo production: modified synthetic oviduct fluid (SOFaaci) and synthetic oviduct fluid–bovine embryo 1 (SOF-BE1) media. All samples were immediately frozen or stored after oocyte selection for further evaluation as previously described [22,23,30].

### 2.3. Embryo production

*In vitro* production (IVP) of bovine embryos was performed as previously described [22,31], unless otherwise noted. Only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were used. Selected oocytes were *in vitro* matured (IVM) by incubation at 38.5 °C in 5% CO<sub>2</sub> in air with saturated humidity for 24 hours. Drops containing 90 µL of TCM 199 with Earle salts and L-glutamine (Gibco, Invitrogen Co., Grand Island, NY, USA), supplemented with 5 mg/mL BSA (fatty acid free), 0.2 mM sodium pyruvate, 5 mg/mL LH (Lutropin-V; Bioniche Co., Belleville, ON, Canada), 1 mg/mL FSH (Folltropin; Bioniche Co.), 100 µg/mL of streptomycin sulfate, and 100 IU/mL of penicillin (Gibco, Invitrogen Co.), containing 20 to 30 oocytes each, were placed in petri dishes and covered with mineral oil. At the end of the *in vitro* maturation period, groups of 20 to 30 oocytes were fertilized with Percoll-purified sperm [32], at the concentration of 2 × 10<sup>6</sup> sperm/mL in 90 µL-containing drops of fertilization media covered with mineral oil. Fertilization occurred in modified Tyrodes for *in vitro* fertilization medium [32] supplemented with 6 mg/mL BSA (fatty acid free; Sigma A8806), 0.2 mM pyruvate, 30 µg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine, 1.8 µM epinephrine, 100 µg/mL streptomycin sulfate, and 100 IU/mL penicillin (Gibco, Invitrogen Co.). Oocytes and sperm were incubated under the same conditions as IVM for approximately

18 hours. After incubation, presumptive zygotes were denuded by repeated pipetting and randomly transferred to culture plates under drops (20–30 structures per drop) containing 90  $\mu\text{L}$  of SOFaaci or SOF-BE1, covered with mineral oil. Note that the experimental model included, by design, two distinct, but commonly used, culture media conditions aiming to control for the confounding effect of the culture media composition. Stage-specific lipidome signatures identified during early embryo development should represent temporal, developmentally modulated changes. *In vitro* culture was performed at 38.5 °C in an atmosphere of 5%  $\text{CO}_2$  and 5%  $\text{O}_2$  with the balance  $\text{N}_2$ . The SOFaaci [33] medium was composed by 107.63 mM NaCl, 7.16 mM KCl, 1.19 mM  $\text{KH}_2\text{PO}_4$ , 1.51 mM  $\text{MgSO}_4$ , 1.78 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.35 mM sodium lactate, 25.0 mM  $\text{NaHCO}_3$ , 0.2 mM sodium pyruvate, 0.20 mM L-glutamine, 45.0  $\mu\text{L}/\text{mL}$  basal medium eagle essential amino acids (50 $\times$ ), 5.0  $\mu\text{L}/\text{mL}$  minimum essential medium nonessential (100 $\times$ ), 0.34 mM trisodium citrate, 2.77 mM myo-inositol, 10  $\mu\text{g}/\text{mL}$  phenol red, and supplemented with 2.5% fetal calf serum (FCS), 5 mg/mL BSA (fatty acid free; Sigma A8806), 100  $\mu\text{g}/\text{mL}$  streptomycin sulfate, and 100 IU/mL of penicillin (Gibco, Invitrogen Co.). The SOF-BE1 composition was 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM  $\text{KH}_2\text{PO}_4$ , 0.49 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.17 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.30 mM sodium lactate, 25.07 mM  $\text{NaHCO}_3$ , 0.4 mM sodium pyruvate, 1.00 mM alanyl glutamine, 20  $\mu\text{L}/\text{mL}$  basal medium eagle essential amino acids (50 $\times$ ), 10  $\mu\text{L}/\text{mL}$  minimum essential medium nonessential (100 $\times$ ), 0.50 mM trisodium citrate, 2.77 mM myo-inositol, supplemented with 4.0 mg/mL BSA (fatty acid free; Sigma A8806), and 25.0  $\mu\text{L}/\text{mL}$  gentamicin sulfate, as previously described [34]. Embryos remained in this condition until Day 7 post-insemination. Immature oocytes (–24 hpi; denuded by repeated pipetting) and embryos at the two-cell stage (32–40 hpi), eight- to 16-cell embryos (72 hpi), morula (120 hpi), and blastocyst (168 hpi) were collected for lipid profiling by MALDI-MS and staining with Nile Red for cytoplasmic lipid droplets visualization and for quantitative polymerase chain reaction (qPCR) evaluation of lipid metabolism-related genes. A total of 2000 oocytes were used in seven IVP replicates to achieve all the required embryos for the experiments. Cleavage (80.0% and 80.3%) and blastocyst (38.3% and 25.0%) rates were similar for SOFaaci and SOF-BE1 media, respectively. Embryos of each development stage were recovered randomly from each IVP replicate from individual drops used just to collect a specific stage avoiding the use of the same drop to produce early and than later stages.

#### 2.4. Cytoplasmic lipid droplets staining

A sample of denuded immature oocytes ( $n = 8$ ) and of each early embryo development stage (two-cell embryos, eight- to 16-cell embryos, morula, and blastocyst stages;  $n = 5$ –9 per stage, from each of the two culture media) were randomly selected during experimental replications and stained with Nile Red (Molecular Probes, Eugene, OR, USA), a fluorescent dye for intracellular lipid droplets, as previously described [35,36]. Denuded oocytes and embryos were washed in a solution of 0.1% (wt/vol) polyvinylpyrrolidone in phosphate-buffered saline solution

(PVP-PBS) and fixed in 4% (vol/vol) formaldehyde in PVP-PBS solution during 1 hour. A stock solution was prepared by dissolving Nile Red in DMSO at a concentration of 1 mg/mL and stored at –20 °C. Oocytes and embryos were stained with a work solution of 15  $\mu\text{g}/\text{mL}$  of Nile Red in PVP-PBS solution during 1 hour in the dark at room temperature. Final concentration was obtained by diluting the stock with the PVP-PBS. Stained structures were washed again in PVP-PBS and mounted in 10  $\mu\text{L}$  glycerol on cover slips and examined under a fluorescence microscope to take a digital photograph at the equatorial plane of the oocyte and embryo at  $\times 400$  magnification in the microscope focus that had the greater number of lipid droplets. Fluorescence intensity (FI) was quantified using ImageJ 1.47 t software (version 1.60\_65, Wayne Rasband; National Institutes of Health, Washington DC, USA). Oocyte and embryo color pictures were converted to a gray-scale image and oocyte/embryos were delimited (using the freehand selection tool on ImageJ) to obtain area ( $\mu\text{m}^2$ ) and gray intensity mean (arbitrary unit). Background was removed (considering where no embryo was present to be zero to compensate for variation of the UV lamp intensity) by subtracting the value where no embryo was present from the measurement of the oocyte and embryo, and the gray intensity mean per area was calculated (arbitrary units/ $\mu\text{m}^2$ ) and presented as FI (mean/area).

#### 2.5. Lipid profiling by MALDI-MS

##### 2.5.1. Sample preparation

Denuded immature oocytes ( $n = 6$  pools containing five immature oocytes each) and early development stage embryos (two-cell embryos, eight- to 16-cell embryos, morula, and blastocyst;  $n = 11$ –17 single embryos/stage) were washed five times in drops of PBS solution and stored in microtubes containing 2 to 4  $\mu\text{L}$  of PBS at –80 °C until analysis. Samples were thawed by pipetting 100  $\mu\text{L}$  of a solution of 1:1 (vol/vol) methanol/ultrapure water (ACS/HPLC grade; Burdick and Jackson, Muskegon, MI, USA, and Millipore, Bedford, MA, USA) into the microtube and washed five times in the same solution. Each embryo or pool of five oocytes was placed on a unique spot on the MALDI target plate under the stereomicroscope. Samples were allowed to dry at room temperature, and their location was recorded on a map. Just before analysis, 1  $\mu\text{L}$  of 1.0 M of 2,5-dihydroxybenzoic acid diluted in pure methanol was deposited on each target spot to cover the embryos, and the spots were allowed to dry at room temperature.

##### 2.5.2. MALDI-MS data acquisition

MALDI-MS and matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) data were acquired in reflectron mode using an Autoflex III MALDI time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with smart beam laser technology. The mass spectra were acquired in the  $m/z$  700 to 1200 range, in positive ion mode, by averaging 1500 consecutive laser shots with a frequency of 200 until signals in the region of interest were observed and then disappeared due to the consumption of the sample. MALDI-MS/MS was manually performed by increasing the

collision energy until extensive dissociation of the precursor ion was observed. Argon was used as the collision gas. FlexAnalysis 3.3 software (Bruker Daltonics) was used to check the mass spectra. The most intense ions, which were clearly distinct from noise after the exclusion of isotopic peaks, were considered from each spectrum and used as the starting point to search for  $m/z$  values corresponding to lipids.

### 2.5.3. Lipid assignment

MALDI-MS/MS (laser-induced fragmentation technique [LIFT]) analysis was performed to confirm the structure of lipid species that were significant for experimental group differentiation. To increase the intensity of the signal and simplify the isolation of the parent ions, a pool of 10 oocytes/embryos per group was placed on each spot. The LIFT data, data previously obtained in the literature [21,22,30,37,38], and two lipid databases (<http://lipidsearch.jp> or [www.lipidmaps.org](http://www.lipidmaps.org)) were used to assign PL (PC, PE, and SM) and triacylglycerol species.

### 2.6. Determination of the relative abundance of mRNA transcripts for ACSL3, ELOVL5, and ELOVL6

Relative mRNA abundance fluctuation of target genes involved in lipid metabolism throughout embryo development was evaluated in a total of four and eight biologic replicates (pools) for immature oocytes and early embryos, respectively. Pools of 20 (for immature oocytes), 15 (for two-cell embryos, eight- to 16-cell embryos, and morula), and seven (for blastocyst) structures were submitted to total RNA extraction using the RNeasy Micro kit (Qiagen, Mississauga, ON, Canada) followed by DNase treatment (Qiagen) and reverse transcription (Oligo-dT primer and SuperScript III; Applied Biosystems, Foster City, CA, USA). The cDNA equivalent of two embryos was used for the evaluation of transcript abundance for *ACSL3*, *ELOVL5*, and *ELOVL6* and housekeeping genes *GAPDH*, *SDHA*, and *YWHAZ* in a 7300 Real Time PCR System (qPCR; Applied Biosystem) using GoTaq qPCR Master Mix (Promega, Madison, WI, USA). PCR conditions were as follows: 2 minutes at 95 °C followed by 40 cycles each of 15 seconds at 95 °C, and 1 minute at 60 °C. Data were analyzed using the standard curve method [39]. The reference gene was the geometric mean of the Ct values of *GAPDH*, *SDHA*, and *YWHAZ* [40]. The *ACSL3* (5'CCACAGACTTTAGCAGATCAGTCTT3' and 5'CGATCCATGATTTCCGGGAC3'), *ELOVL5* (5'GGCCACATCAGCA GCTTTTC3' and 5'ACGATGTGGTTCAGAGGCTG3'), and *ELOVL6* (5'TCAGTTGCCCTTGGCTTTCA3' and 5'CCCAGCTCAAGAACTT CGGT3') primer pairs were used. Others primer pairs (*GAPDH*, *SDHA*, and *YWHAZ*) were published previously [40].

### 2.7. Statistical analysis

For cytoplasmic lipid droplets content and transcript abundance evaluation, data were analyzed with ANOVA using the generalized linear mixed model (GLIMMIX) procedure with the SAS statistical software package (SAS Institute Inc., Cary, NC, USA). Sources of variation in the model included development stage (immature oocytes, two-cell embryos, eight- to 16-cell embryos, morula, and

blastocyst), culture media (SOFaaci and SOF-BE1), replicate, and first other interaction. All factors, except replicate, were considered to be fixed effects. If the ANOVA was significant, means were separated using Tukey's test. Logarithmic transformation was applied to qPCR data to improve normality. The data are reported as untransformed least-squares means  $\pm$  SEM.

For mass spectrometry lipid profiles analysis, multivariate and univariate statistical models were used. Missing values were replaced by the half of the minimum positive value in the data obtained from the preprocessing procedure. The intensity values of each peak across multiple spectra were autoscaled (mean-centered and divided by the standard deviation of each variable) to give the same importance to every  $m/z$  value. Partial least square discriminant analysis (PLS-DA) was performed using the MetaboAnalyst 2.0 [41] to show the relationship between variance in the data and differences among embryo development stage (immature oocytes, two-cell embryos, eight- to 16-cell embryos, morula, and blastocyst) samples. The differentially expressed lipid species between early embryo development stages were identified using ANOVA followed by Tukey's test. Hierarchical clustering of the differentially expressed lipid species was performed using Euclidean distances and Ward linkage to show relationship between samples and features.

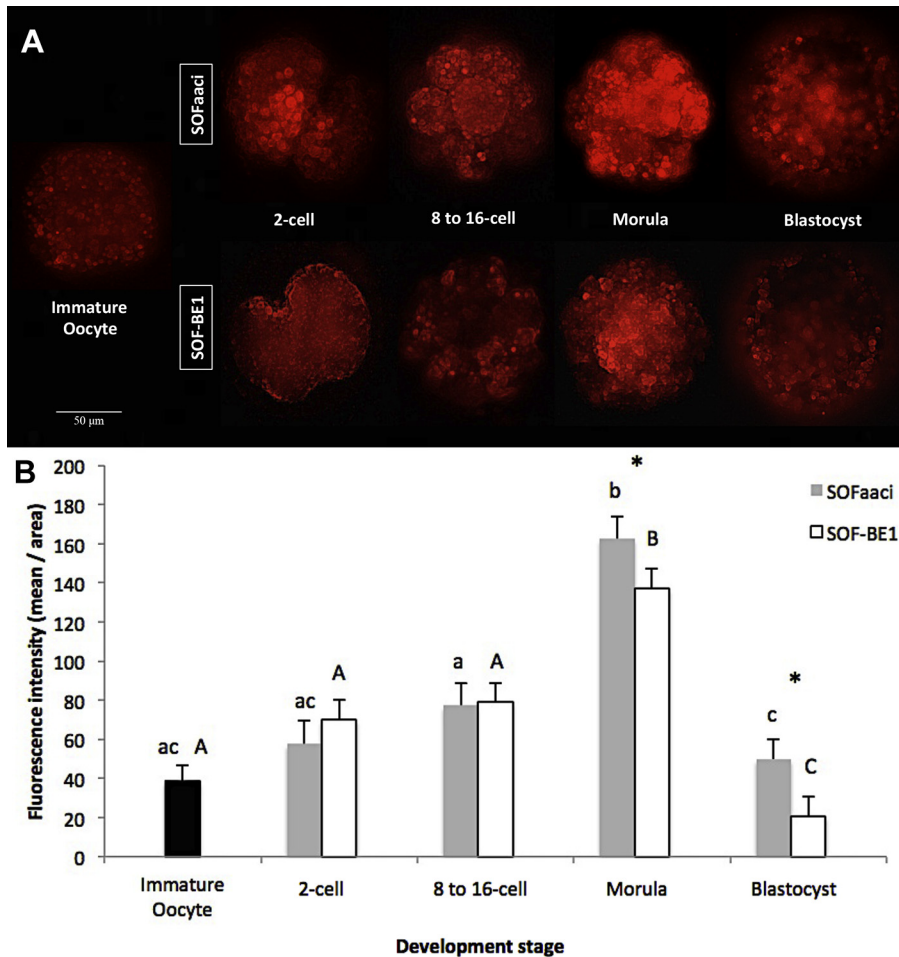
## 3. Results

### 3.1. Cytoplasmic lipid droplets content

The cytoplasmic lipid droplets were homogeneously distributed in the cytoplasm of oocyte and early embryos (Fig. 1A). There were a culture media and a culture media by development stage interaction effects ( $P < 0.05$ ). Lipid content increased ( $P < 0.05$ ) at morula stage compared with other stages, followed by a decrease ( $P < 0.05$ ) at blastocyst stage in both culture media (Fig. 1B). The impact of the culture media was observed only at the morula and blastocyst stages, in which SOFaaci medium caused an increase ( $P < 0.05$ ) in the cytoplasmic lipid content compared with SOF-BE1 (Fig. 1B).

Immature oocytes presented similar lipid content level ( $P > 0.05$ ) compared with embryos of two-cell and eight- to 16-cell stages cultured in both SOFaaci- and SOF-BE1 media. However, lipid content was reduced ( $P < 0.05$ ) in SOF-BE1-derived blastocysts compared with oocytes and two-cell and eight- to 16-cell stages (Fig. 1B). SOFaaci-derived blastocysts had lower ( $P < 0.05$ ) cytoplasmic lipids compared with SOFaaci-derived eight- to 16-cell embryos. Although there were a culture media and culture media by development stage interaction effects ( $P < 0.05$ ) on cytoplasmic lipid droplets content evaluation (Fig. 1B), no remarkable culture media and culture media by developmental stage interaction effects ( $P > 0.05$ ) were identified in lipid profile and transcript-level evaluations; therefore, data were combined for all further analysis and only development stage results are presented.

Additionally, we have also analyzed the cytoplasmic lipid droplet content corrected by the median cell number of each development stage [42]: two cells (two-cell embryos' lipid content FI divided per two), eight to 16 cells (eight- to



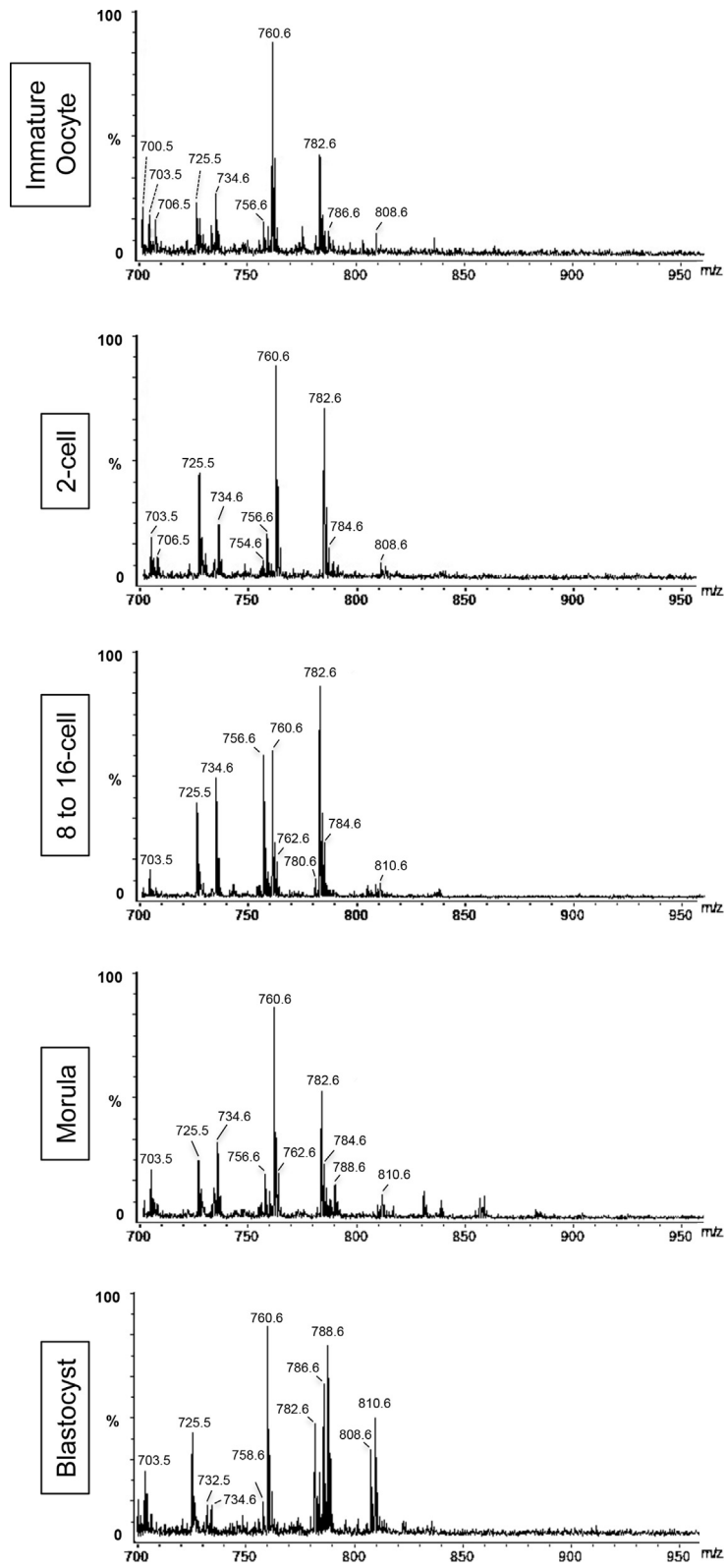
**Fig. 1.** (A) Representative images of cytoplasmic lipid droplets labeled with Nile Red at immature oocyte, and two-cell, eight- to 16-cell embryos, morula, and blastocyst stages of development cultured in SOFaaci or SOF-BE1 media. (B) Stage-specific lipid content during early bovine embryo development expressed by mean fluorescence intensity per area (least-squares mean  $\pm$  SEM). Values without common lowercase (within SOFaaci group) or uppercase (within SOF-BE1 group) letters differ significantly ( $P < 0.05$ ;  $n = 5$ –9 per group) throughout embryo development compared with the immature oocyte. Asterisks (\*) represent significant differences ( $P < 0.05$ ) between SOFaaci and SOF-BE1 media within development stage. SOFaaci, modified synthetic oviduct fluid; SOF-BE1, synthetic oviduct fluid–bovine embryo 1. (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

16-cell embryos' lipid content FI divided per 12), morula (morula's lipid content FI divided per 48), and blastocyst (blastocyst's lipid content FI divided per 132). Cytoplasmic lipid droplet content was increased ( $P < 0.05$ ) at immature oocytes ( $58.4 \pm 3.5^{\text{aA}}$ ; lowercase and uppercase letters compared with SOFaaci and SOF-BE1 derived embryos, respectively) compared with other stages produced using SOFaaci ( $28.8 \pm 4.1^{\text{b}}$ ,  $6.4 \pm 3.8^{\text{c}}$ ,  $3.4 \pm 3.8^{\text{c}}$ ,  $0.4 \pm 4.4^{\text{c}}$ ; respectively for two-cell embryos, eight- to 16-cell embryos, morula, and blastocyst stage) and SOF-BE1 ( $35.0 \pm 4.5^{\text{B}}$ ,  $6.6 \pm 4.1^{\text{C}}$ ,  $2.9 \pm 3.3^{\text{C}}$ ,  $0.2 \pm 4.4^{\text{C}}$ , respectively, for two-cell embryos, eight- to 16-cell embryos, morula, and blastocyst stage). Two-cell embryos had more ( $P < 0.05$ ) lipid droplets content than eight- to 16-cell embryos, morula, and blastocyst. There was no difference ( $P > 0.05$ ) in the lipid content among eight- to 16-cell embryos, morula, and blastocyst. Moreover, the lipid content of SOFaaci and SOF-BE1-derived embryos were not different ( $P > 0.05$ ). How the cytoplasmic lipid content of role embryos (data not

corrected by the cell number) were close related with mRNA levels of lipid metabolism-related genes and agrees with the literature that serum-containing media increase embryo lipid content [14,20,43], it was decided to considerate these results for the data interpretation and comprehensive lipid analysis during early embryo development.

### 3.2. Stage-specific MALDI-MS lipid signatures in early bovine embryo development

No remarkable culture media and culture media by developmental stage interaction effects ( $P > 0.05$ ) were identified in lipid profile; therefore, data were combined for all further analysis and only development stage results are presented. PL and TAG structures are named by the lipid class abbreviation (PC, PE, SM, and TAG) followed by the total number of carbons and double bonds attached to the glycerol backbone and separated by a colon. Lipid structures were attributed on the basis of LIFT data (described



**Fig. 2.** Representative MALDI-MS in the positive ion mode for stage-specific lipid profiles of immature oocytes (n = 6) and *in vitro*-produced embryos (n = 11–17); the intensity presented is relative to the most intense ion pick, and each pick represents one phospholipid.

subsequently), previous MALDI-MS lipid profile studies [21,22,30,37,38], and through consulting two lipid databases (<http://lipidsearch.jp> or [www.lipidmaps.org](http://www.lipidmaps.org)). Figure 2 displays a representative lipid profile of each development stage. Note that a mixture of isomers is frequently observed for a single lipid ion.

PLS-DA analysis shows that early embryo development stages can be resolved through their MALDI-MS profiles by group clustering in the two-dimensional PLS-DA score plots (Fig. 3A). Slight overlaps between immature oocytes, and embryos of two-cell and eight- to 16-cell stages can be observed (Fig. 3A).

For the three-dimensional PLS-DA plot, however, early embryo development stages were separated, with even more pronounced group individualization and greater deviation starting at eight- to 16-cell stage and advancing to morula and blastocyst stages (Fig. 3B). Supplementary Table 1 lists the discriminated significant lipids from early development stages as indicated by the PLS-DA analysis.

Hierarchical clustering analysis (Supplementary Fig. 1) of the acquired MALDI-MS spectra also suggested that the averaged peak intensities extracted from each set data are well separated and distinguishable between early embryo development stages. These data also allow following the fluctuations of each lipid species during embryo development and, in combination with the differentially expressed lipid species (Fig. 4), suggests putative biomarker peaks for dynamic lipid changes during early bovine embryo development.

Differentially abundant lipid species between immature oocytes and early embryo development stages were identified (Fig. 4). Only significant changes in the lipid profiles during embryo development were annotated.

In immature oocytes, the relative abundance of protonated PC (30:0) and PC (30:3) and sodiated PE (38:5) were increased ( $P < 0.05$ ) compared with other development stages (Fig. 4; please find in Supplementary Table 1 each lipid assignment and its respective  $m/z$ ). In contrast, the relative abundances of protonated SM (16:0), PCe

(32:0), PC (36:2), PC (36:1), sodiated SM (16:0), protonated PC (38:5) and/or sodiated PC (36:2), and protonated PC (38:4) and/or sodiated PC (36:1) were reduced ( $P < 0.05$ ) in immature oocytes compared with blastocysts (Fig. 4).

The sodiated PC (32:1) and/or PC (34:4) and protonated PC (30:0) were overexpressed ( $P < 0.05$ ) in two-cell embryos compared with other development stages (Fig. 4). Nonetheless, the protonated PE (38:5) levels started to drop at two-cell stage and remained low until the blastocyst stage (Fig. 4).

At the eight- to 16-cell stage, protonated PC (32:0) and PC (34:2), sodiated PC (32:0), and sodiated PC (34:2) and/or protonated PC (36:5) presented a greater ( $P < 0.05$ ) relative abundance compared with other stages, whereas the relative intensity of protonated PC (30:0) started to drop at eight- to 16-cell stage and remained low until blastocyst stage (Fig. 4).

At the morula stage, the relative abundances of the protonated and sodiated SM (16:0) started to increase ( $P < 0.05$ ), whereas the protonated and sodiated PC (32:0) levels were increased ( $P < 0.05$ ) at this developmental stage (Fig. 4).

Finally, seven lipids were remarkably more abundant ( $P < 0.05$ ) at the blastocyst compared with the other stages (protonated SM [16:0], PCe [32:0], PC [36:2], PC [36:1]; sodiated [SM, 16:0]; and protonated PC [38:5] and/or sodiated PC [36:2]; and protonated PC [38:4] and/or sodiated PC [36:1]; Fig. 4). The protonated and sodiated PC (32:0) were less abundant ( $P < 0.05$ ) in blastocysts compared with morula and eight- to 16-cell embryos (Fig. 4), whereas the relative intensity of protonated PC (30:0) was reduced ( $P < 0.05$ ) at blastocyst stage compared with immature oocyte and two-cell stage (Fig. 4).

### 3.3. MS/MS characterization of PLs in immature oocytes and embryos

To confirm the structural attributions for lipids on the basis of the literature and database search, lipid ions were subjected to MS/MS via the LIFT-MS technique [44]. The

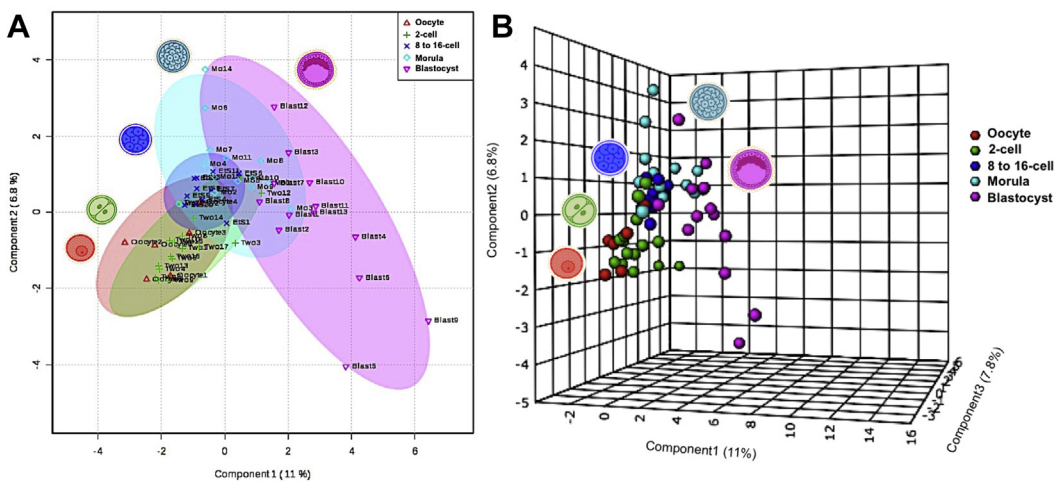
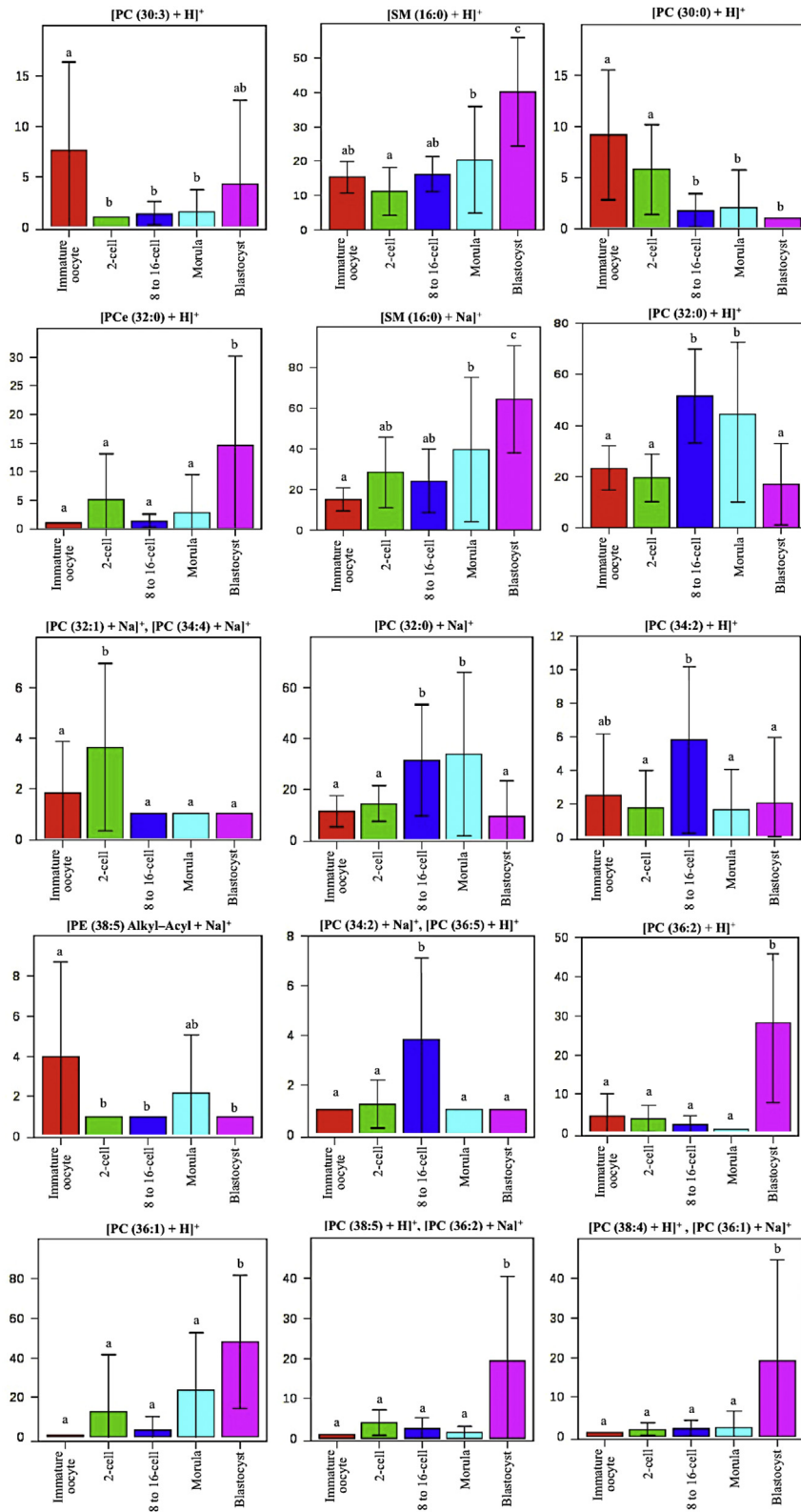


Fig. 3. Two-dimensional (A) and three-dimensional (B) partial least-squares discriminant analysis plots from MALDI-MS data of stage-specific lipid profiles of *in vitro*-produced bovine embryos;  $n = 6$  to 17 per group.





**Fig. 4.** Relative intensities of differentially expressed phospholipids of stage-specific *in vitro*-produced bovine embryos. PC, phosphatidylcholines; PC with a lower case e refer to plasmalmyl subspecies; PE, phosphatidylethanolamine; SM, sphingomyelins; n = 6 to 17 per group.

MS/MS fragmentation of lipid ions can provide class structural attributions. Our MS/MS results were compatible with those reported in previous studies [21,22,30,37,38,45]. The loss of a neutral of 59 Da corresponds to neutral trimethyl amine [N(CH<sub>3</sub>)<sub>3</sub>], whereas loss of 124 Da is related to the cyclophosphane ring (C<sub>2</sub>H<sub>5</sub>O<sub>4</sub>P). The fragment ion of *m/z* 147 corresponds to sodiated cyclophosphane and that of *m/z* 184 to monoprotonated dihydrogen phosphate choline (C<sub>5</sub>H<sub>15</sub>PO<sub>4</sub>N) (Supplementary Fig. 2).

3.4. Relative abundance of mRNA transcripts for ACSL3, ELOVL5, and ELOVL6 in early bovine embryo development

No remarkable culture media and culture media by developmental stage interaction effects (*P* > 0.05) were identified in transcript-level evaluations. Therefore, data were combined for all further analysis and only development stage results are presented. Levels of mRNA of target genes related with metabolic pathways that activate fatty acids, synthesize complex lipids, and elongate fatty acids (ACSL3, ELOVL5, and ELOVL6) were evaluated to gain further insight into lipid metabolism. Relative ACSL3, ELOVL5, and ELOVL6 mRNA abundances increased (*P* < 0.05) at morula stage compared with other stages, followed by a decrease (*P* < 0.05) at blastocyst stage (Fig. 5).

ACSL3 mRNA level was, however, reduced (*P* < 0.05) in blastocysts compared with oocytes, two-cell embryos, eight- to 16-cell embryos, and morula stages (Fig. 5), whereas mRNA relative abundance of ELOVL5 was reduced (*P* < 0.05) in blastocysts compared with two-cell, eight- to 16-cell, and morula stages (Fig. 5). ELOVL6 mRNA level was only reduced in blastocysts compared with morula stage. Other comparisons between different development stages of each target genes mRNA level were not different (*P* > 0.05).

3.5. Comprehensive lipid analysis during early bovine embryo development

Lipid profiles started to deviate with the increased abundances of specific membrane lipid species (protonated

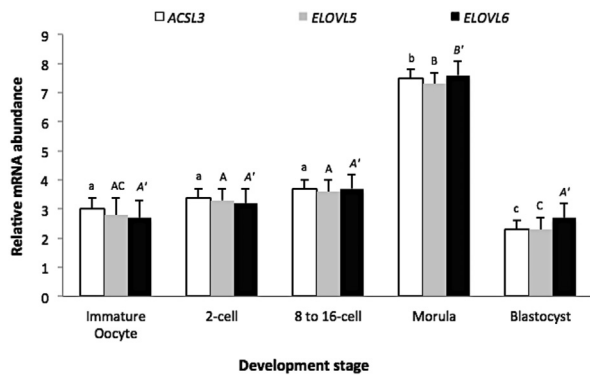


Fig. 5. Stage-specific relative abundance of mRNA transcripts for ACSL3, ELOVL5, and ELOVL6 during early bovine embryo development (least-squares mean ± SEM). Values without common lowercase (within ACSL3), uppercase (within ELOVL5), and in italic accent uppercase (within ELOVL6) letters differ significantly (*P* < 0.05; *n* = 4–8 per group) throughout embryo development compared with the immature oocyte.

PC [32:0] and PC [34:2], sodiated PC [32:0], and protonated PC [36:5] and/or sodiated PC [34:2]) in eight- to 16-cell embryos (Table 1).

Basal levels of ACSL3 mRNA were presented until eight- to 16-cell stage, followed by an increase at morula, and a subsequent drop at blastocyst stage (Table 1). In the same way, relative ACSL3 mRNA level was associated with the cytoplasmic lipid content fluctuation throughout development, presenting a close relationship between lipid droplets accumulation and the relative abundance of mRNA transcript for ACSL3, revealing a putative cytoplasmic lipid accumulation biomarker (Table 1).

Relative ELOVL5 and ELOVL6 mRNA abundance increased at morula stage followed by a decrease at blastocyst stage. It is well documented that ELOVLs elongate saturated, monounsaturated, and polyunsaturated fatty acids after ACSL activation. ELOVL5 and ELOVL6 higher expression preceded the increase of a series of lipid species at blastocyst stage containing saturated, monounsaturated, and polyunsaturated elongated fatty acids with 16, 18, and 20 carbons attached to the three-carbon backbone of PL (Table 1), indicating potential markers of embryo PL fatty acid elongation. Furthermore, after ACSL3, ELOVL5, and ELOVL6 expression drop, cytoplasmic lipid content has decreased (Table 1), suggesting that lipid droplets were consumed (i.e., lipid droplets fatty acids were activated by ACSLs) for the synthesis of complex lipids, such as membrane PL containing elongated fatty acids at the blastocyst stage.

In addition, the early embryo lipid profile dynamics revealed putative stage-specific biomarker peaks, independent of environmental conditions (i.e., culture media), which can be readily associated with key stage-dependent biologic process during embryogenesis (Fig. 4, and Table 1), e.g., oocyte maturation and fertilization (immature oocyte

Table 1

Comprehensive results of notable fluctuations in cytoplasmic lipid droplets content, relative mRNA transcripts of ACSL3, ELOVL5, and ELOVL6, and lipid profiles levels in eight- to 16-cell embryos, morula, and blastocyst.

Variable/development stage	Eight- to 16-cell embryos	Morula	Blastocyst
Cytoplasmic lipid droplets content	Basal	↑	↓
Relative mRNA transcript levels			
ACSL3	Basal	↑	↓
ELOVL5	Basal	↑	↓
ELOVL6	Basal	↑	Basal
MALDI-MS lipid profiles			
[SM (16:0) + H] <sup>+</sup>	Basal	↑	↑
[PCe (32:0) + H] <sup>+</sup>	Basal	Basal	↑
[SM (16:0) + Na] <sup>+</sup>	Basal	↑	↑
[PC (32:0) + H] <sup>+</sup>	↑	↑	Basal
[PC (32:0) + Na] <sup>+</sup>	↑	↑	Basal
[PC (34:2) + H] <sup>+</sup>	↑	Basal	Basal
[PC (34:2) + Na] <sup>+</sup>	↑	Basal	Basal
[PC (36:5) + H] <sup>+</sup>	Basal	Basal	↑
[PC (36:2) + H] <sup>+</sup>	Basal	Basal	↑
[PC (36:1) + H] <sup>+</sup>	Basal	Basal	↑
[PC (38:5) + H] <sup>+</sup>	Basal	Basal	↑
[PC (36:2) + Na] <sup>+</sup>	Basal	Basal	↑
[PC (38:4) + H] <sup>+</sup>	Basal	Basal	↑
[PC (36:1) + Na] <sup>+</sup>	Basal	Basal	↑

Arrows indicate elevated (↑) or reduced (↓) relative abundance levels in relation to the other group. Basal level was defined as the most common abundance value between groups.

PL variations; protonated PC [30:0] and PC [30:3], sodiated PE [38:5]); fertilization and cleavage (two-cell embryo PL variations; protonated PC [30:0], and sodiated PC [32:1] and/or PC [34:4]); cleavage and embryonic genome activation (eight- to 16-cell embryo PL variations; protonated PC [32:0] and PC [34:2], sodiated PC [32:0], and sodiated PC [34:2] and/or protonated PC [36:5]); compaction and blastocyst formation (morula PL variations; protonated and sodiated SM [16:0] and PC [32:0]); and differentiation, blastocoel formation and expansion, and hatching (blastocyst PL variations; protonated SM [16:0], PCe [32:0], PC [36:2], PC [36:1], sodiated SM [16:0], protonated PC [38:5] and/or sodiated PC [36:2], and protonated PC [38:4] and/or sodiated PC [36:1]).

#### 4. Discussion

We provide a comprehensive lipid analysis of membrane PL dynamics during early bovine embryo development in a high-throughput manner association through mass spectrometry, cytoplasmic lipid droplets content, and target qPCR transcripts monitoring. The amount of cytoplasmic lipid droplets increased at morula followed by a decreased at blastocyst stage during preimplantation period that was closely related with the *ACSL3* mRNA levels. Significant differences in the lipid profiles according to the developmental stage of *in vitro*-produced bovine embryos were identified, sustaining that PL undergoes dynamic changes during embryonic development, which can be associated with key biologic process during embryogenesis. Increased *ELOVL5* and *ELOVL6* mRNA levels at morula stage preceded an increased abundance of membrane PL containing elongated saturated, monounsaturated, and polyunsaturated fatty acids with 16, 18, and 20 carbons at blastocyst stage.

A series of studies have described the composition, in terms of fatty acids, TAG and lipid droplets content, oocytes, and embryos of many species [14,16,29]. Structural and composition changes in the lipid profiles throughout development and their potential relationship with cellular and molecular mechanisms governing mammalian embryo preimplantation development are, however, still poorly understood.

Somatic cells take up free fatty acids that are esterified and stored in lipid droplets mainly as TAG [46]. A major biologic function of these lipids is the storage of metabolic energy [16,17]. In the present work, lipid droplets were found to be homogeneously distributed in immature oocyte/embryo cells, agreeing with previous descriptions [16,22]. The amount of cytoplasmic lipid droplets increased at morula stage concomitant with the raise of *ACSL3* mRNA levels. It seems that there is a combination of fat accumulation and genome activation, which may start to take place at the eight- to 16-cell stage (for a review, see Sirard [47]).

It has already been described that lipid droplet levels decrease during embryo development [48–50]. Indeed, lipid content was found to drop at blastocyst stage. A possible hypothesis to the decrease in overall fat content during blastulation is their consumption, mainly by trophoblastic cells, via  $\beta$ -oxidation [16,22], to support an increased energy demand during blastocyst formation, expansion of the blastocoel, and hatching [16,51].

However, we cannot oversimplify the role of TAG when only considering them as an energy source. It may be fair to propose the initial role of TAG in cellular metabolism as a precursor for the synthesis of PL [52]. Indeed, we have observed a great number of overexpressed PL lipid species at blastocyst stage, which is corroborated by the reduction of cytoplasmic lipid droplets content and greater membrane lipid demand synthesis at this stage to allow an active increase in embryo cell number. An interesting observation is the occurrence of these events after the overexpression of transcripts coding for the fatty acid activation- and elongation-associated enzymes (*ACSL3*, *ELOVL5*, and *ELOVL6*) at morula stage, which could be linked to the activation of lipid droplets fatty acids in preparation for the synthesis of complex lipids, such as PL membrane-containing elongated fatty acids (Table 1).

The influence of different culture environmental conditions was only observed when cytoplasmic lipid droplets were assessed, at morula and blastocyst stages, in which SOFaaci medium increased cytoplasmic lipid droplets content compared with SOF-BE1. In the present experiment, two SOF-based media for embryo culture, a classical one (SOFaaci [33]), commonly used worldwide and supplemented with fetal calf serum, and an SOF-based medium (SOF-BE1 [34]), with a formulation that allows the absence of fetal calf serum supplementation that is recognized by its detrimental impact in the embryo lipid content and cryosurvival, was used [20,43]. Despite the similarity between these two SOF-based culture media, they have some major differences in their composition including the absence of FCS, increased concentration of glutamine (5 $\times$ ), pyruvate (2 $\times$ ), and essential and nonessential amino acids (2.25 and 2 $\times$ , respectively) in SOF-BE1 compared with SOFaaci. May be, this increased lipid content observed in the SOFaaci-derived embryos is associated with the increased mRNA levels of *ACSL3*, after genome activation, that favors serum's fatty uptake by embryo cells, as observed previously in human placental trophoblast cells [26]. The exact mechanism regulating the accumulation of cytoplasmic lipid droplets in oocytes/embryos is still unknown. It is, however, well documented that FCS media culture supplementation increases embryo lipid content [20].

As already described, lipid droplets or endoplasmic reticulum membranes present great abundance of *ACSL3* protein [53]. *ACSL3* isoform has a highest affinity for its preferred substrates: laurate (C12:0), myristate (C14:0), arachidonate (C20:4), and eicosapentaenoic acid (C20:5) [54]. In addition, each *ELOVL* enzyme isoform also has its substrate preference, e.g., *ELOVL5* elongates palmitoleic (C16:1), oleic (C18:1), linolenic (C18:3), stearidonic (C18:4), arachidonic (C20:4), and eicosapentaenoic (C20:5) acids [27]; and *ELOVL6* elongates long chain-saturated (palmitic acid; C16:0) and monounsaturated (palmitoleic acid; C16:1) fatty acids [28]. These long chain fatty acids are key components of membrane PL [27].

Cells and intracellular compartments are separated from their environment by membranes. Biologic membranes vary regarding the associated proteins and their lipid composition. Thousands of lipid molecules have already been described in the lipidome of cellular membranes, each with

its own specific properties indicating that lipids do not only work simply as passive structural membrane components [55]. Understanding the lipid profile of membranes is important to address the mechanisms governing mammalian preimplantation development. Changes in the lipid structural composition of the membranes can be readily detected by mass spectrometry [22].

MALDI-MS has proved to be a powerful tool in lipidomics [56], generating PL (mainly PC, PE, and SM in the positive ion mode) and TAG profiles with simple sample manipulation and data interpretation. Through MALDI-MS, several major PL were identified for each experimental group. As expected, some overlaps were observed between oocytes and embryo development stages, especially during the first cleavage stages (immature oocyte and embryos at the two-cell and eight- to 16-cell stages), whereas with the progression of development, greater deviation starts at eight- to 16-cell stage and advances to morula and blastocyst stages. The shift in membrane lipid profile observed at the eight- to 16-cell stage could be related to the acquisition of regulatory molecules of fat metabolism, after embryonic genome activation, which resulted in lipid profile differences at morula and blastocyst stage due to lipid membrane remodeling and specialization, sustained by the variations of mRNA levels of lipid metabolism-associated genes (*ACSL3*, *ELOVL5*, and *ELOVL6*). Potentially, this specific period could be an eligible moment for functional experiments to modulate embryo lipid metabolism to favor or inhibit some specific lipid species.

Confirming the hypothesis of lipid membrane fluctuation throughout embryo development, the number of carbons, fatty acid saturation, and class composition for the PL are actively varying. Phospholipids were found, therefore, to undergo pronounced dynamic structural changes during embryonic development. Our study was focused on the characterization of PL, but significant variations in TAG profiles were also identified.

Remarkable changes in numerous lipid species were observed at immature oocytes and preimplantation embryos. Note that all these observed differences were independent of culture media, which further validates the relevance of the identified molecules regardless environmental conditions during early embryo development. The early embryo lipid profile dynamics revealed putative biomarker peaks for lipid stage-specific induced changes which can be readily associated with key biological process related with important events during *in vitro* and *in vivo* embryogenesis period (e.g., oocyte maturation, fertilization, cleavage, embryonic genome activation, compaction, differentiation, blastocoel formation and expansion, and hatching). Whether these specific changes in membrane PL profiles are involved with each embryo biologic processes shall make for interesting functional studies. We have to be cautious to extrapolate all these findings for *in vivo* retrieved embryos because of the differences between cultures systems that is reflected to lipid profiles peculiarities between *in vitro*- and *in vivo*-produced embryos [22]. Commonly the IVP methodologies use culture conditions where embryos grow in medium with oil overlay inside incubators instead of the interior of oviduct and uterus. Further investigation is required to determine whether

these differentially expressed PL species are truly involved in each event of embryogenesis period.

Previous studies have described variations in lipid species of oocytes/embryos undergoing vitrification [38,57], mass spectrometry imaging [7], cultured under different protein source and atmospheric conditions [21], between different subspecies [22], and between *in vivo*- and *in vitro*-derived embryos [8,22].

In the present work, we have observed variations of unsaturation degree of PL fatty acyl residues, fatty acyl chain length, and differences in PL class composition. Additionally, *ELOVL5* and *ELOVL6* overexpression preceded an increased abundance of a series of lipid species at blastocyst stage containing saturated, monounsaturated, and polyunsaturated elongated fatty acids with 16, 18, and 20 carbons attached to the three-carbon backbone of PL.

In conclusion, this study reports characteristic lipidome signatures of a comprehensive lipid analysis involving cytoplasmic lipid droplets staining, membrane PL's mass spectrometry evaluation, and qPCR lipid metabolism-related genes monitoring of immature oocytes and preimplantation embryos. Our results suggested that the cytoplasmic lipid droplets content and mRNA transcript levels of *ACSL3*, *ELOVL5*, and *ELOVL6* in immature oocytes and preimplantation embryos are associated with the chemical structure of their membrane lipids, discriminating dynamic changes during early embryonic development. The data presented here represents an initial step to assess the role of specific lipid species in important events of embryogenesis and identified some putative lipid metabolism biomarkers for cytoplasmic lipid droplets content (*ACSL3* expression) and fatty acid elongation (*ELOVL5* and *ELOVL6* expression) in bovine embryos.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.theriogenology.2016.03.025>.

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**Supplementary Table 1**

Significant phospholipids and triglycerides indicated via MALDI-MS of individual early bovine embryos.

<i>m/z</i>	Lipid ion (carbons:unsaturation)
700.5	[PC (30:3) + H] <sup>+</sup>
703.5	[SM (16:0) + H] <sup>+</sup>
706.5	[PC (30:0) + H] <sup>+</sup>
720.5	[PCe (32:0) + H] <sup>+</sup>
725.5	[SM (16:0) + Na] <sup>+</sup>
727.5	[SM (d34:0) + Na] <sup>+</sup>
732.5	[PC (32:1) + H] <sup>+</sup>
734.6	[PC (32:0) + H] <sup>+</sup>
742.6	[PCp (34:2) + H] <sup>+</sup> , [PCe (34:3) + H] <sup>+</sup>
746.6	[PE (36:1) + H] <sup>+</sup>
748.6	[PE (38:6) alkenyl-acyl + H] <sup>+</sup>
753.6	[SM (18:0) + Na] <sup>+</sup>
754.6	[PC (32:1) + Na] <sup>+</sup> , [PC (34:4) + Na] <sup>+</sup>
756.6	[PC (32:0) + Na] <sup>+</sup>
758.6	[PC (34:2) + H] <sup>+</sup>
760.6	[PC (34:1) + H] <sup>+</sup>
762.6	[PC (34:0) + H] <sup>+</sup>
768.6	[PE (42:4) + H] <sup>+</sup> , [PCp (36:3) + H] <sup>+</sup>
774.6	[PE (38:5) alkyl-acyl + Na] <sup>+</sup>
780.6	[PC (34:2) + Na] <sup>+</sup> , [PC (36:5) + H] <sup>+</sup>
782.6	[PC (36:4) + H] <sup>+</sup> , [PC (34:1) + Na] <sup>+</sup>
784.6	[PC (34:0) + Na] <sup>+</sup>
786.6	[PC (36:2) + H] <sup>+</sup>
788.6	[PC (36:1) + H] <sup>+</sup>
798.6	[PC (34:1) + K] <sup>+</sup>
808.6	[PC (38:5) + H] <sup>+</sup> , [PC (36:2) + Na] <sup>+</sup>
810.6	[PC (38:4) + H] <sup>+</sup> , [PC (36:1) + Na] <sup>+</sup>
829.5	TAG (50:4)
836.6	[PC (40:5) + H] <sup>+</sup>
855.7	TAG (50:1)
857.6	TAG (50:0)
881.7	TAG (52:2)
883.7	TAG (52:1)
903.7	TAG (54:5)

Identification based on LIFT data, literature [21,22,37,38], and two lipid databases (<http://lipidsearch.jp> or [www.lipidmaps.org/](http://www.lipidmaps.org/)).

Abbreviations: PC, phosphatidylcholines; PC with a lower case *e* and/or *p* refer to plasmanyl and plasmenyl subspecies, respectively; PE, phosphatidylethanolamine; SM, sphingomyelins; TAG, triacylglycerol.