Breed specific factors influence embryonic lipid composition: comparison between Jersey and Holstein

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Abstract

Some embryos display better survival potential to cryopreservation than others. The cause of such phenotype is still unclear and might be due to cell damage during cryopreservation, resulting from over-accumulation and composition of lipids. In cattle embryos, *in vitro* culture conditions have been shown to impact the number of lipid droplets within blastomeres. So far, the impact of breed on embryonic lipid content has not yet been studied. In this study were compared the colour, lipid droplet abundance, lipid composition, mitochondrial activity, and gene expression of *in vivo* collected Jersey breed embryos which are known to display poor performance post-freezing and *in vivo* Holstein embryos which have good cryotolerance. Even when housed and fed in the same conditions, Jersey embryos were found to be darker and to contain more lipid droplets than did Holstein embryos, and this was correlated with lower mitochondrial activity. Differential expression of genes associated with lipid metabolism and differences in lipid composition were found. These results show genetic background can impact embryonic lipid metabolism and storage.

Introduction

Over the years, dairy milk production has increased steadily due to several factors, including improved management, nutrition and breeding program (Lucy 2001). Combined with advances in assisted reproduction technology, such as artificial insemination, superovulation, embryo freezing and transfer, conventional breeding has contributed the most to accelerating genetic gain (Bousquet *et al.* 1998). In the field of embryo transfer, a constant increase in the demand for frozen rather than fresh embryos is currently observed (Stroud 2011). Freezing allows storage and transportation of embryos and better management of donors and recipients, and is commercially advantageous since genetics can be exchanged easily without exporting livestock. However, this technology is still challenging and damage to the embryo occurs frequently. Several factors contribute to the success rate of embryo transfer, including production (*in vivo* or *in vitro*) (Crosier *et al.* 2001; Fair *et al.* 2001; Hasler 2001; Rizos *et al.* 2003), culture media composition (Yamashita *et al.* 1999; Hasler 2001; Abe *et al.* 2002b; Abe and Hoshi 2003; Rizos *et al.* 2003), species (Massip 2001; Van Soom *et al.* 2003; Guignot 2005), embryo quality (Lindner and Wright 1983; Hasler 2001; Van Soom *et al.* 2003; Guignot 2005) and lipid content (Yamashita *et al.* 1999; Abe *et al.* 2002b; Abe and Hoshi 2003).

In the dairy industry, cattle breeds differ considerably. The Holstein is recognized for providing milk in the greatest volume, while the Jersey is popular because of the high fat index of the milk. The high fat content of Jersey milk suggests that the biochemical or physiological makeup of this breed may involve differences in lipid metabolism (Beaulieu and Palmquist 1995). It has also been observed that Jersey embryos do not

tolerate freezing very well. Steel and Hasler showed that Jersey embryos frozen in either ethylene glycol or glycerol produced significantly fewer pregnancies than did Holstein embryos (Steel and Hasler 2004). It has been suggested that the lower tolerance of Jersey embryos might be associated with a high intracellular lipid content, causing increased damage to cells during cryopreservation.

An inverse correlation between cytoplasmic lipid content and tolerance of freezing or cooling has been observed among embryos cultured in media containing serum (Abe *et al.* 1999; Yamashita *et al.* 1999; Hasler 2001; Abe *et al.* 2002b; Reis *et al.* 2003). Changes in mitochondrial structure and function in association with accumulation of intracellular lipid have been detected in embryos cultured in such media (Kruip *et al.* 1983; Dorland *et al.* 1994; Thompson *et al.* 1995; Sata *et al.* 1999; Crosier *et al.* 2001; Abe *et al.* 2002b; Abe and Hoshi 2003; Rizos *et al.* 2003; Plourde *et al.* 2012). Since mitochondria are not static organelles but vital determinants of normal early embryonic development (Dumollard *et al.* 2007) and located where ATP must be supplied at high levels (Tarazona *et al.* 2006), these changes should be expected to reduce embryo quality.

So far, Jersey and Holstein breed effects on embryonic lipid metabolism have not been documented. We hypothesized that embryonic lipid content are different between Jersey and Holstein due to their intrinsic differences in lipid management. This project was conducted with animals housed and fed under the same conditions to isolate the genetic component associated with embryonic lipid composition. Stage specific embryos were compared on the basis of their lipid content, composition, metabolism potential and gene

expression. This work provides a different perspective to embryonic lipid composition by addressing the need to account for breed specific differences.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

Production and recovery of embryos in vivo

Non-lactating healthy Holstein (n = 4) and Jersey (n = 4) cows were housed and fed under the same conditions. Animals were kept for a year and were repetitively submitted to *in vivo* embryo collection. All animals were collected at least seven times. Embryos were staged and graded according to the IETS scores. Only morula and early blastocysts graded 1 and 2 were used in this study. All breed comparisons were done on samples matched for developmental stage and grade quality.

Embryos were obtained from L'Alliance Boviteq Inc. (Saint Hyacinthe, Québec, Canada). All animals used in this study were handled following the guidelines provided by the Canadian Council on Animal Care. These guidelines are strictly followed by L'Alliance Boviteq who provided all the tissues and samples. The study did not require handling animals on university premises.

The cows received a super-ovulating treatment: Follicles of diameter larger than 8 mm were aspirated on day 8–12 post-oestrus. Administration of FSH (Folltropin-V, Bioniche Animal Health) was begun 36 hours later (twice daily in doses decreasing from

60 mg to 20 mg for a total of 400 mg over four days). Prostaglandin F2α analogue (Estrumate, Intervet, Kirkland, QC, Canada) was administrated in doses of 500 µg with each of the two final FSH injections to initiate luteolysis. Cows appearing oestrus at 36 h after the final FSH/Estrumate injection were inseminated twice with pooled semen (12 and 24 h later). On day 6 after insemination, embryos were recovered by uterine flushing and categorized according to the IETS system. Fresh embryos were needed for some assays while other analyses allowed freezing of the embryos which were then washed three times in PBS, placed into 0.5-ml microtubes in a minimum volume, snap-frozen and conserved at -80°C.

Characterization of lipid droplets and active staining of mitochondria

Mitochondria in fresh blastocyst embryos (n = 15 per breed) were stained with 300 nM of the active dye CMX-rosamine (Mitotracker Red, Molecular Probes, Eugene, OR, USA) in synthetic oviduct fluid (SOF) for 40 minutes at 38 °C in 5 % CO_2. The dye showed strong sensitivity to the mitochondrial membrane potential, mitochondrial protein (thiol groups) and exhibited better retention and much more even distribution compared to other dyes, due to high co-localization with cytochrome C oxidase (Poot *et al.* 1996). The fluorescence excitation wavelength was 594 nm and emission was read at 608 nm. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), which uncouples mitochondrial membrane potential, was employed as a negative control to set the background which was used as a mean of calibration between runs. In parallel experiments, embryos selected randomly were treated with 100 nM CCCP and incubated for 15 min at 38 °C in a humidified 5 % CO_2 atmosphere before adding CMX-rosamine.

Following staining with CMX-rosamine, the embryos were immersed in 3 μg/mL of the lipid-specific dye 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (Bodipy 493/503, Molecular Probes, Eugene, OR, USA) in SOF for 10 minutes. To label nuclei, embryos were incubated with 1 μg/mL of Hoechst blue dye 33342 in SOF for 10 min at room temperature, washed three times in SOF and mounted on microscope slide coverslips.

Confocal microscopy

Bright field, confocal and epifluorescence images were acquired using a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada) with a 60x/ 1.20 water-immersion objective. Bright field images of morula embryo morphological phenotype were recorded in grey scale photos with the same settings to estimate colour (dark or pale) based on the IETS system. Confocal images of the whole lipid volume of each embryo were acquired with a z-stack, space by 0.5 μm, from a first section at the bottom of embryo next to the coverslip. This total thickness of optical sections (20 μm) was sufficient to obtain homogeneity of the Bodipy 493/503 fluorescence, as established in preliminary experiments. The optical sections were recorded with 512 x 512 pixel resolution. The respective excitation and emission wavelengths were as follows: Bodipy (488 nm, 515-530 nm), and the Mitotracker Red (555nm, 605-675nm). All the settings were similar for all samples. Mitochondrial activity was the recorded as an epifluorescence image of CMX-rosamine dye in grey scale and carried out using Nikon

TE2000 microscope quipped with epifluorescence illumination and appropriate filters, which were G-2a for the Mitotracker Red.

Image Analysis

The intensity of CMX-rosamine fluorescence was measured using the mean grey scale in IMAGE J software (Abramoff *et al.* 2004). Results are expressed in arbitrary units (AU) as the mean fluorescence intensity of all samples within a group. Measurements of the number and the volume of lipid droplets in embryos of each section were obtained using the plugin LIPID DROPLET COUNTER of IMAGE J software (Abramoff *et al.* 2004). The minimal droplet size threshold was set at 5 pixels (which represents 0.5 μm^2) to overcome false-positive counts due to background pixels. The mean volume of lipid droplets in this size range was calculated in femtolitres (1 fL = 10^{-15} litres).

Isolation of total DNA and RNA

Additional blastocysts (n = 5 embryos per breed) were used for total genomic DNA and RNA, extracted simultaneously using the AllPrep DNA/RNA Micro Kit (Qiagen, Mississauga, ON, Canada) according the manufacturer's instructions. Genomic DNA was used for mitochondrial DNA quantification and total RNA was reverse-transcribed and analysed using quantitative RT-PCR (qRT-PCR).

Quantification of mitochondrial DNA

Individual embryos (n = 10 per breed) were used to quantify mitochondrial DNA (mtDNA) using a quantitative PCR (qPCR) method with genomic DNA. The 12S rRNA gene (GenBank accession number J01394) was selected as a mitochondrial target and Mx1 gene (GenBank accession number AY340484) as a nuclear target (Table 1). The mtDNA and nuclear DNA (nDNA) were used to calculate the relative concentration of mtDNA in each embryo, which was expressed as the mtDNA/nDNA ratio. The LightCycler 2.0 (Roche Diagnostics) was used for qPCR reactions. The reaction mixture (20 µL) contained 0.5 µL of each primer solution (0.25 µM), 1.2 µL of 1.5 µM $MgCl_2$, 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Laval, QC, Canada) and 5 µL of DNA sample. The average DNA concentration of each sample was 0.00335 ng/µL. The following cycling conditions were applied for amplification: initial denaturation at 95 °C for 10 min followed by 50 cycles of 95 °C for 5 sec, 5 sec (12S rRNA) at 58 °C or 60 °C (Mx1), followed by 72 °C for 20 sec and 76 °C (12S rRNA) or 85 °C (Mx1) for 5 sec. The presence of amplicons was verified using melting curve analysis: Following the last amplification cycle, the internal temperature of the LightCycler was rapidly increased to 94 °C then decreased to 72 °C for 30 s, followed by a slow increase to 94 °C at a rate of 0.1 °C per s, with continuous fluorescence reading. Quantification of mtDNA and nDNA copy numbers was performed based on a standard curve, which was based on the linear relationship between the crossing point cycle values and the logarithm of the starting copy number.

189 *Differential gene expression in Holstein and Jersey embryos*

190 Total RNA of individual blastocysts (n = 4 per breed) was extracted using
191 PicoPure RNA kit (Molecular Devices, Downingtown, PA, USA) according the
192 manufacturer's instructions and DNase I digestion (Qiagen). The quality and
193 concentration of the extracted RNA was measured using a model 2100 Bioanalyzer
194 (Agilent Technologies, Palo Alto, CA, USA) with the RNA PicoLab Chip (Agilent
195 Technologies). Only RNA of very good quality (RIN over 8) was used for amplification.

196 Purified RNA was amplified in two rounds using T7 RNA polymerase and a
197 RiboAmp HSPlus Amplification Kit (Life Sciences, Foster City, CA, USA). RNA
198 concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop,
199 Wilmington, DE, USA). Antisense-RNA (aRNA) samples were labelled with Cy3 or Cy5
200 using the Universal Linkage System (ULS) kit (Kreatech Diagnostic, Amsterdam,
201 Netherlands) and 825 ng of labelled aRNA were hybridized on Agilent EmbryoGENE
202 slides (Robert *et al.* 2011) in a two-colour dye-swap design in a hybridization oven for 17
203 h at 65 °C. To have a transcriptomic individual composition, a total of four biological
204 replicates were done using single blastocyst from each cow breed. A technical dye swap
205 replicate was also performed for a total of eight hybridizations. Microarray slides were
206 then washed and scanned using a PowerScanner (Tecan, Männedorf, Switzerland) and
207 analysed with Array-Pro Analyzer software (MediaCybernetics, Bethesda, MD, USA).

208 Microarray data were pre-processed as described in previous studies (Plourde *et al.*
209 2012), using Lowess intra-array and quantile inter-array normalizations. Statistically
210 significant variations were detected using Limma (Flexarray, Génome Québec, Montréal,

211 Canada). Differences in gene expression were considered significant when a cut-off
212 adjusted p-value < 0.01 and change of at least 1.2-fold were obtained. Pathway analyses
213 and downstream exploitation of gene lists were carried out using Ingenuity Pathway
214 Analysis Software Version 8.6 (Ingenuity Systems Inc., Redwood City, CA, USA).

215

216 *Quantitative RT-PCR validation*

217 Validation of microarray results was performed using qRT-PCR on additional
218 embryos (n=5 per breed). RNA was reverse-transcribed using the qScript cDNA
219 SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) with an oligo-dT to prime the
220 reaction as per the manufacturer's recommendations. Primers were designed for
221 candidates (ADIPOR2: Adiponectin receptor 2, LPIN1: Lipin-1, LPIN2: Lipin-2, and
222 ELVOL5: ELOVL fatty acid elongase 5) using the Primer3 Web interface
223 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized at IDT
224 (Coralville, IA, USA). The reaction mixture was composed of the LightCycler FastStart
225 DNA Master SYBR Green I kit components (Roche Diagnostics, Laval, QC, Canada)
226 and real-time measurements were performed in a LightCycler 2.0 apparatus (Roche
227 Diagnostics). Our real-time PCR amplification procedure has been described previously
228 in detail (Gilbert *et al.* 2010). Genebank accession number, primer sequences, annealing
229 temperatures, and product size are shown in Table 1.

230 For quantification, real-time PCR was performed as described previously
231 (Bermejo-Alvarez *et al.* 2010). Each pair of primers was tested for reaction efficiency,
232 and the comparative cycle threshold (ΔCT) method was then used to quantify differences

in transcript levels as described by Schmittgen and Livak (Schmittgen and Livak 2008) . Quantification was normalized (ΔΔCT) to the endogenous control (beta-actin to account for cell number). Change in the relative level of gene expression of the target was calculated as $2^{-\Delta\Delta CT}$.

Analysis of lipid profile by MALDI-MS

Lipid profiles of intact cattle embryos were done as described in the literature (Ferreira *et al.* 2010) with some modifications. Briefly, embryos (n = 7 for each breed) collected at the morula stage were washed three times in PBS solution and stored at -80 °C in 0.5 mL micro-tubes containing 2-4 µL of PBS until analysis. Samples were thawed in 100 µL of 50 % (v/v) methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA) in ultrapure water (Millipore, Billerica, MA, U.S.A.) and washed three times in this solution. Each embryo was placed on a single spot on the Matrix-assisted laser desorption/ionization (MALDI) target plate. Samples were allowed to dry at room temperature, and their locations were recorded. Prior to analysis, 1 µL of 1.0 M 2,5-dihydroxybenzoic acid diluted in methanol was placed on each target spot to cover the embryo, and the spots were allowed to dry at room temperature.

Mass spectra were recorded in reflector mode using an AB SCIEX 4800 MALDI TOF/TOF TM instrument (AB Sciex, Concord, Ontario, Canada) equipped with a Nd:YAG laser operating at 355 nm and 200 Hz. Laser intensity remained fixed for all the analyses. External calibration was performed and mass accuracy was better than 50 ppm. MS spectra were acquired between 700-1000 Da. The sample plates received 10 V and

60–90 s of laser shots on the sample spot region, until signals in that region disappeared due to ablation of the sample. MALDI-MS data were acquired by impact energy until extensive break-up of the precursor ion. Argon was used as the collision gas. Spectra were centred and aligned using MassLynx 4.0 software (Waters, Manchester, UK). From each spectrum, after exclusion of isotopic peaks, the most intense ions were considered as the starting point for searching m/z values corresponding to lipids. After attribution, only the m/z values that were clearly above background levels were included in the principal component analysis, which was performed using Pirouette v.3.11 (Infometrix Inc., Woodinville, WA, USA). The laser-induced fragmentation technique (LIFT) polar lipid database obtained from previous studies (Ferreira *et al.* 2010; Sudano *et al.* 2012) was used to identify lipids in this study.

Statistical analyses

The number and mean volume of lipid droplets in embryos were tested using Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA). Student's t-test was applied for comparisons between breeds. Differences were declared significant when $P < 0.05$. Lipid MS profiles, multivariate and univariate statistical models were used as described previously (Ferreira *et al.* 2010; Sudano *et al.* 2012). A first principal component analysis (PCA) was performed using Pirouette v.3.11 (Infometrix, Inc.). Based on the MALDI-MS results the ions with significant signals intensities over background value were selected for analysis using Student's t-test in order to verify them for both breeds.

Results

Abundance of lipid droplets makes embryos appear darker

The overall appearance of Holstein and Jersey embryos at the morula stage is shown in Figure 1. The blastomere cytoplasm was darker in Jersey embryos than in Holstein embryos, which can be classified as "pale" based on the IETS system.

Lipid droplets, considered as a fatty acid storage reservoir in cells, were identified and quantified in embryos of both breeds, using the neutral lipid stain (BODIPY) according to the Aardema protocol (Aardema *et al.* 2011). As demonstrated in Figure 2(A and B), differences in lipid droplet abundance are observed between the breeds. Jersey embryos have a higher number of droplets and these are of lower average volume compared to Holstein ($p < 0.05$, C and D).

Lipid droplet numbers in Jersey embryos are related to lower mitochondrial activity

The red dye CMX-rosamine is commonly used to assess mitochondrial survival or functional mitochondria (Poot *et al.* 1996). As shown in Figure 3, Holstein and Jersey embryos (A and B) seem to have a similar mitochondrial distribution and no difference in the mtDNA/nDNA ratio (Figure 3D) was detected. However, the fluorescence intensity was greater in Holstein than in Jersey morula ($7{,}893 \pm 23$ AU; $p < 0.05$), indicating higher mitochondrial activity (Figure 3C).

Gene expression differentials between the Holstein and Jersey breeds

298 To evaluate the differences between the embryos of these breeds, a large-scale
299 transcriptomic analysis was performed using a microarray. Among the 37,238 transcripts
300 represented on the microarray slide, only 83 protein-coding genes were expressed with
301 differentials greater than 1.2 ($p < 0.05$), suggesting that the embryos of both breeds are
302 highly similar. The differentially expressed genes were analysed by Ingenuity Pathways
303 Analysis (IPA, www.ingenuity.com). Among the different biological functions thus
304 identified, we focused on lipid metabolism genes that might explain the observed
305 differences in lipid content and mitochondrial activity. The analysis revealed that fatty
306 acid release, oleic acid oxidation, palmitic acid uptake and acylglycerol synthesis were
307 the most significant categories of differential lipid metabolism function (data not shown).
308 Validation of the microarray results was performed using qRT-PCR on four (ADIPOR2,
309 LPIN1, LPIN2, ELVOL5) selected genes related to lipid metabolism (Table 1). As
310 observed in the microarray analysis, the expressions of these selected genes have a
311 positive trend (between 0.09 and 0.1) in Holstein than in Jersey embryos (Figure 4).

312

313 *Differences in Jersey and Holstein lipid profiles detected by MALDI-MS*

314 In combination with the microarray, a lipid composition analysis was also performed.
315 Mass spectrometry provides fast and simple means of determining lipid profiles. Matrix-
316 assisted laser desorption/ionization mass spectrometry (MALDI-MS) can provide a lipid
317 fingerprint of a single intact cattle embryo directly, in particular of phospholipids such as
318 phosphatidylcholines and sphingomyelins (Ferreira *et al.* 2010; Sudano *et al.* 2012). The
319 most significant lipids thus identified, based on MALDI-MS, are specified in Table 2.

320 The lipid profiles of embryos of each breed are shown in Figure 5(A and B).
321 Measurement of lipid ion abundance revealed that protonated sphingomyelin (16:0),
322 phosphatidylcholine (32:0, 34:2) and sodiated sphingomyelin (16:0) were significantly
323 higher ($P < 0.05$) in Jersey than in Holstein embryos (Figure 5C). Principal component
324 analysis of the MALDI-MS data revealed a spatial arrangement in two distinct clusters
325 corresponding to the cattle breeds, with no overlap (Figure 6).
326

327 **Discussion**

328 The capacity for tolerating cryopreservation is an important criterion for embryo
329 quality in the commercial setting. Species such as pigs and humans and some breeds of
330 cattle (such as Jersey) do not tolerate this procedure very well. In the dairy industry, the
331 Jersey breed is recognized for production of high-fat milk. Breeders regard the Jersey
332 cow as versatile and well suited to any production system. However, the success rate of
333 embryo cryopreservation is low for this breed (around 43.0 %) compared to the Holstein
334 breed (approaching 55.8 %) (Steel and Hasler 2004), thus limiting the utilization of the
335 Jersey cow. This problem is believed to be associated with the high lipid content of the
336 Jersey embryo.

337 The colour of the blastomere cytoplasm is considered an accurate indicator of embryo
338 quality (Lindner and Wright 1983; Thompson *et al.* 1995; Sata *et al.* 1999; Abe *et al.*
339 2002b; Van Soom *et al.* 2003; Guignot 2005) and also appears to be a predictor of
340 embryo tolerance of cryopreservation (Yamashita *et al.* 1999; Fair *et al.* 2001; Massip
341 2001; Abe *et al.* 2002b; Van Soom *et al.* 2003; Guignot 2005). However, evaluation of
342 this criterion is subjective, and many factors, including breed, can influence coloration.

Previous studies comparing dairy breeds to beef breeds have shown that Holstein embryos obtained *in vivo* were darker than Belgian blue (Van Soom *et al.* 2003; Leroy *et al.* 2005). It was suggested that differences in embryo colouring likely involve factors other than genetics, such as physiological status associated with high milk production. Comparing embryos of different subspecies, Visintin *et al* found that Nellore (*Bos indicus*) embryos were "pale" compared to Holstein embryos. Embryo quality was found associated with the number of lipid droplets, which was higher in Holstein blastomeres. (Visintin *et al.* 2002).

Lipid concentration is a parameter used to estimate post-fertilization competence in bovine oocytes (Aardema *et al.* 2011) and survival of cryopreservation by bovine embryos (Lindner and Wright 1983; Yamashita *et al.* 1999; Fair *et al.* 2001; Van Soom *et al.* 2003; Guignot 2005). However, this criterion of selection has not been studied in any thorough comparison of breeds. Sudano *et al*. reported that the lipid content is higher in Simmental (*Bos taurus*) embryos than in Nellore embryos (Sudano *et al.* 2012) .

Our results confirmed that blastomeres of Jersey embryos are darker in colour, due mainly to the abundance of lipid droplets, as suggested by Steel and Hasler (Steel and Hasler 2004). However, average lipid droplet volume was higher in Holstein than in Jersey embryos. The superior performance of cryopreserved Holstein embryos in terms of pregnancy rate suggests that the number of lipid droplets in the embryo has a greater impact than has lipid droplet volume on the success of embryo cryopreservation.

Several reports have concluded that there is a close relationship between lipid droplets in cells and mitochondrial activity. In mammalian oocytes, a close spatial

association and hence metabolic relationship between mitochondria and lipid droplets has been reported (Kruip *et al.* 1983; Hyttel *et al.* 1986; Dorland *et al.* 1994; Sturmey *et al.* 2006). It is interesting that the darker cytoplasm observed in bovine embryos produced *in vitro* in several studies appears related to lipid uptake from the serum added to the culture medium and to be a consequence of impaired mitochondrial function (Dorland *et al.* 1994; Thompson *et al.* 1995; Abe *et al.* 1999; Sata *et al.* 1999; Reis *et al.* 2003; Plourde *et al.* 2012). In the present study, Jersey embryos produced *in vivo* had more numerous lipid droplets, due apparently to lower mitochondrial activity. This is in agreement with Visintin *et al*., who reported a stronger inverse relationship between the number of lipid droplets and the number of mitochondria in Holstein embryos compared to Nellore (Visintin *et al.* 2002), and with the findings of Abe *et al*., who observed fewer mature mitochondria in association with higher lipid droplet number in blastomeres with darker cytoplasm in morula obtained *in vivo* and subsequently classified as embryos of lower quality (Abe *et al.* 2002a; Abe *et al.* 2002b). These latter authors suggested that impaired mitochondrial function, expressed as the number of mature (elongated) mitochondria, implied differences in the metabolism of cytoplasmic lipids by mitochondria, possibly affecting the numbers of lipid droplets present.

Analysis of gene expression using a microarray did not reveal many differences overall between the two dairy cow breeds. However, expression of genes associated with lipid metabolism appears to be influenced by the breed component. ADIPOR2 has been described as a major physiological receptor for adiponectin (ADIPOQ) (Yamauchi *et al.* 2003; Fischer *et al.* 2010), which is an adipocyte-derived hormone that plays an important role in the stimulation of fatty acid oxidation and decreases the triglyceride

388 content of cells (Yamauchi *et al.* 2002; Liu *et al.* 2012; Chen *et al.* 2013). Consistent with
389 our data, Zhou *et al*. reported that absence of the FADIPOQ gene in mouse hepatocytes
390 caused a mitochondrial dysfunction that appeared to contribute to increased lipid droplet
391 accumulation as a result of lower mitochondrial activity (Zhou *et al.* 2008). Genes LPIN1
392 and LPIN2 are members of the lipin protein family, which are key effectors of
393 triglyceride and phospholipid biosynthesis (Reue and Zhang 2008). Recent studies have
394 shown that LPIN1 and LPIN2 modulate lipid droplet size, amount, and fatty acid
395 composition in mammalian cells (Valdearcos *et al.* 2012; Sembongi *et al.* 2013).
396 However, individual effects of lipin genes suggested that LPIN2 deficiency results in an
397 increase in lipid droplet biogenesis (Sembongi *et al.* 2013), which could explain the
398 greater abundance observed in Jersey embryos. The gene ELOVL5 appears to play an
399 important role in the synthesis of long-chain mono-unsaturated and polyunsaturated fatty
400 acids (Inagaki *et al.* 2002; Leonard *et al.* 2002; Gregory *et al.* 2011). It has been shown
401 that ELOVL5 is involved in the elongation of palmitic acid (16:0) into stearic acid (18:0),
402 therefore in modifying the palmitic acid (16:0) content of cell membranes and storage
403 lipids (Inagaki *et al.* 2002). In line with these findings, ELOVL5 appears to play an
404 important role in modifying membrane fluidity by changing lipid content and fatty acid
405 composition (Kim *et al.* 2001; Ferreira *et al.* 2010). This could explain the different
406 embryonic sensitivity to cryopreservation observed between Jersey and Holstein (Steel
407 and Hasler 2004).

408 It has been reported previously that lipid content plays an important role in
409 determining the characteristics of cell membranes and that modifying their physical
410 properties is crucial for successful cryopreservation of bovine embryos (Sata *et al.* 1999;

411 Kim *et al.* 2001). Several methods have been developed to evaluate the lipid profile of
412 embryos. However, a major limiting factor is the amount of biological material available
413 for study. Ferreira *et al*. and Sudano *et al*. nevertheless obtained lipid profiles of embryos
414 with limited quantities of sample, using MALDI-MS (Ferreira *et al.* 2010; Sudano *et al.*
415 2012). As expected, we observed an abundance of positive ions well represented in lipid
416 profiles obtained previously in MALDI-MS studies of *in vivo* bovine embryos (Ferreira *et*
417 *al.* 2010; Sudano *et al.* 2012). It has been suggested that cow breed influences the lipid
418 profile observed in the embryos (Sata *et al.* 1999; Sudano *et al.* 2012). This is consistent
419 with our finding that the lipid profiles of Holstein and Jersey embryos do not overlap.

420 Elevated numbers of lipid droplets have been associated previously with variable
421 abundance of lipid ions known to vary in association with cow breed (Sudano *et al.*
422 2012). Although Jersey embryos contained sphingomyelins (16:0 $+H^+$ and 16:0 $+Na^+$) in
423 abundance, these have been found not to have much impact on embryo cryopreservation
424 (Ferreira *et al.* 2010; Kalo and Roth 2011; Sudano *et al.* 2012). We also noted that Jersey
425 embryo was richer in phosphatidylcholine (32:0 $+H^+$ and 34:2$+H^+$) identified as palmitic
426 (16:0) and linoleic (18:2) fatty acids, as described for *Bos taurus* in a previous
427 report(Sudano *et al.* 2012). It remains unclear how linoleic acid content affects embryo
428 tolerance of cryopreservation. A positive effect of conjugated linoleic acid on
429 cryopreservation of embryos produced *in vitro* has been attributed to reducing the number
430 of lipid droplets in cells (Pereira *et al.* 2007). In contrast, Marei *et al*. reported a negative
431 effect of linoleic acid on cryopreservation of oocytes matured in culture medium, but this
432 effect is dependent on concentration and is reversible (Marei *et al.* 2010). Some studies
433 have shown adverse effects on lipid accumulation and lower tolerance of embryos to

cryopreservation following maturation of bovine oocytes in the presence of palmitic (16:0) and stearic (18:0) acids (Shehab-El-Deen *et al.* 2009; Aardema *et al.* 2011; Van Hoeck *et al.* 2011). Based on these studies, we believe that the ratio of saturated to unsaturated fatty acid is critical for the cryopreservation of Holstein and Jersey embryos. Since the Jersey breed is known to produce milk with higher fat content than Holstein, we hypothesized that the follicle environment of these cows is also lipid enriched. This situation could explain, at least in part, the observed differences in fatty acids composition between Jersey and Holstein embryos. However, this link between follicular environment and embryo composition still needs to be explored. We previously treated *in vitro* produced embryos from Jersey and Holstein genetic background with L-carnitine in order to reduce embryonic lipid content through increased embryonic metabolism (manuscript under revision).The results showed a L-carnitine-induced reduction of lipids in both breeds due to increased mitochondrial activity, with milder variations being measured in Jersey embryos. Taken together, these results show that the breed is an important factor to consider when exploring new or improving *in vitro* production procedures. However, the current consensus surrounding embryo production still relies on the belief that all genetic backgrounds respond similarly to treatments and culture conditions.

It is also known that neutral lipid composition is important for developmental competence. According to several studies (Ferguson and Leese 1999; Kim *et al.* 2001; Aardema *et al.* 2011), neutral lipids supply energy to embryos and are linked to improved developmental competence and early embryonic development. The context of this study is focused on the previous observation that Jersey embryos are more sensitive to freezing

than Holstein and aimed at contrasting these two genetic backgrounds and as such the correlation between neutral lipid composition and developmental competence was not explored.

We have shown that the darker cytoplasm observed in embryos of the Jersey cow breed compared to the Holstein cow breed is indeed due to the accumulation of greater numbers of lipid droplets. We documented for the first time that this accumulation was associated with lower mitochondrial activity that is breed specific. Lipid composition showed significant differences between the two breeds, supporting intrinsic deviations in lipid metabolism between both genetic backgrounds. These results provide clear evidences that under identical management breed differences exist at least at the embryonic lipid composition.

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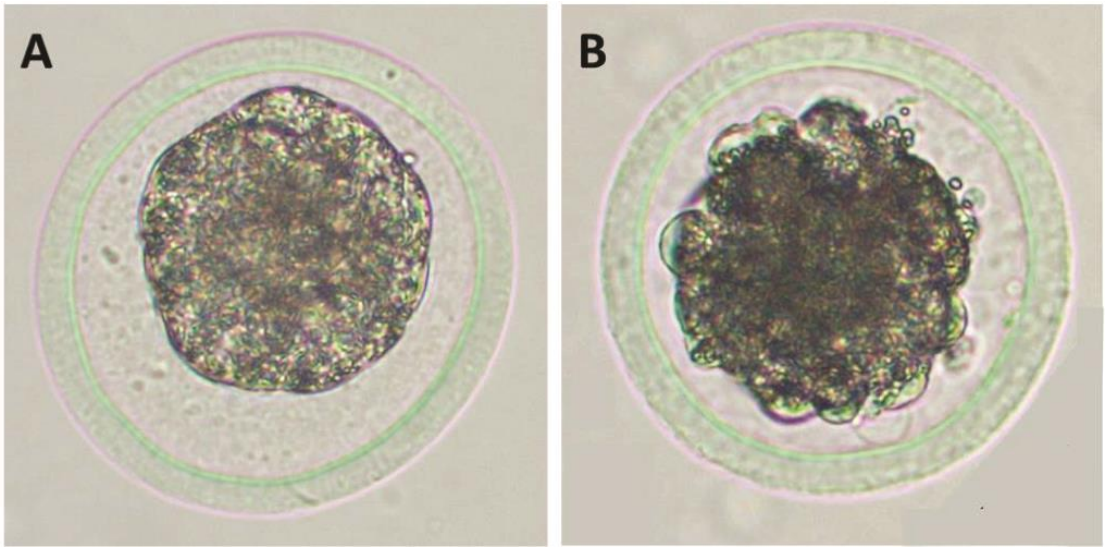


Figure 1. Morphology of bovine embryos (morula stage) collected in vivo six days after insemination. (A) Holstein, categorized as pale, (B) Jersey, categorized as dark. Observed under bright-field microscopy at a magnification of 600x.

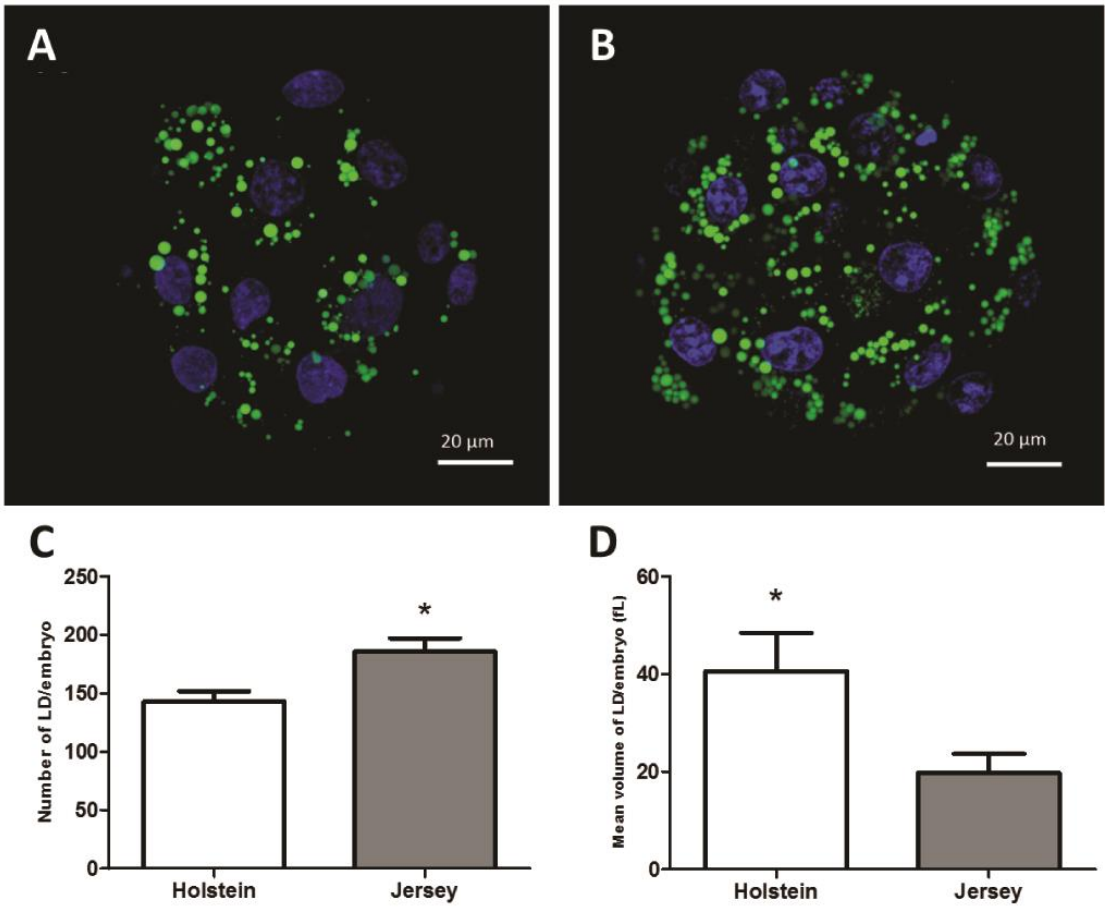
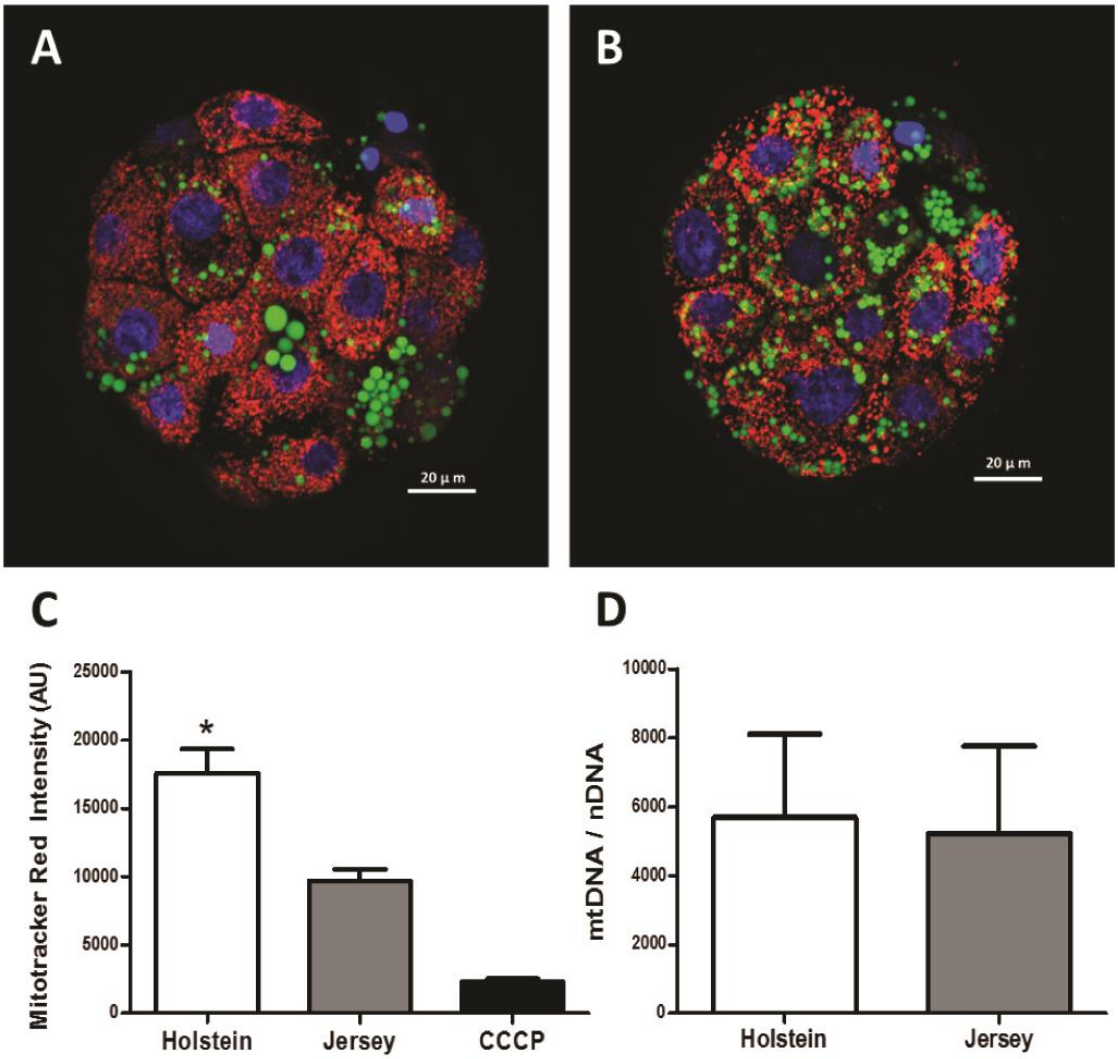


Figure 2. Orthogonal projections of confocal z-stacks representative of lipid droplet content of Holstein (A) and Jersey morula-stage embryos (B) as revealed by staining with Bodipy 493/503 green dye. DNA is stained with Hoechst blue dye. (C) Number of lipid droplets (LD), (D) Lipid droplet mean volume. Values are expressed as mean ± SEM. *Significant difference ($P < 0.05$).



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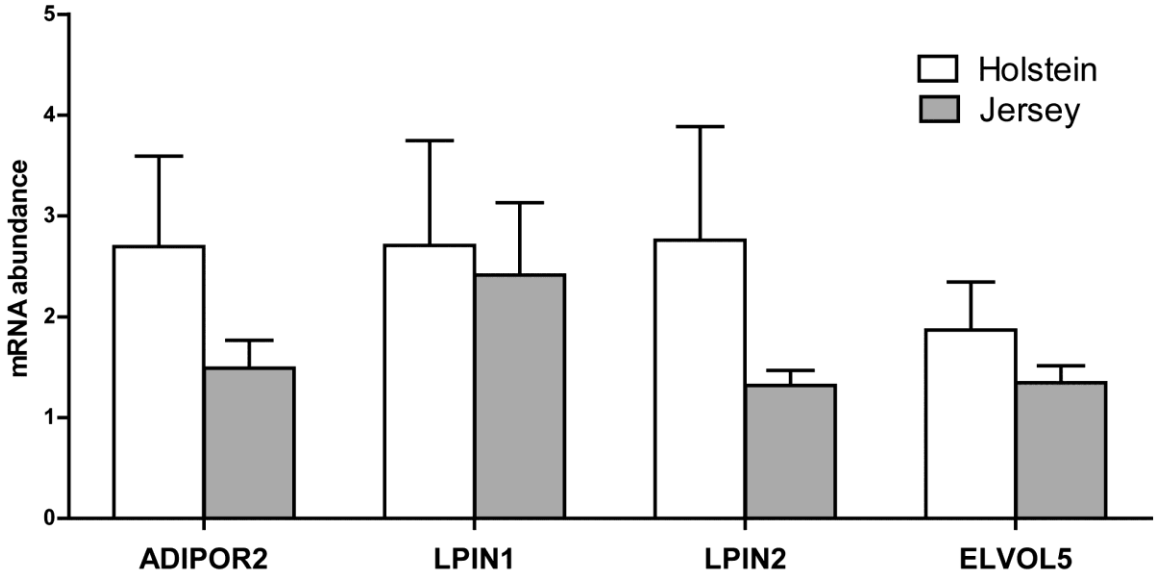
710 **Figure 3**. Confocal microscopic images of Holstein (A) and Jersey (B) embryos (morula
711 stage) obtained in vivo and stained with CMX-rosamine (Mitotracker Red), Bodipy
712 493/503 (green), and Hoechst blue dye 33342 to show respectively active mitochondria,
713 lipid droplets and nuclear DNA. Pictures are orthogonal view reconstructed from a
714 confocal image. (C) CMX-rosamine fluorescence intensity (carbonyl cyanide m-
715 chlorophenylhydrazone (CCCP) was used as a negative control) and (D) Ratio of
716 mitochondrial to nuclear DNA in single blastocysts. Values are mean ± SEM.
717 *Significant difference ($P < 0.05$). AU = arbitrary unit.



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720 **Figure 4**. Quantitative RT-PCR validation of microarray analysis of transcript levels of
721 genes involved in lipid metabolism. Values are mean ± SEM, normalized relative to
722 endogenous β-actin transcripts to account for cell number.

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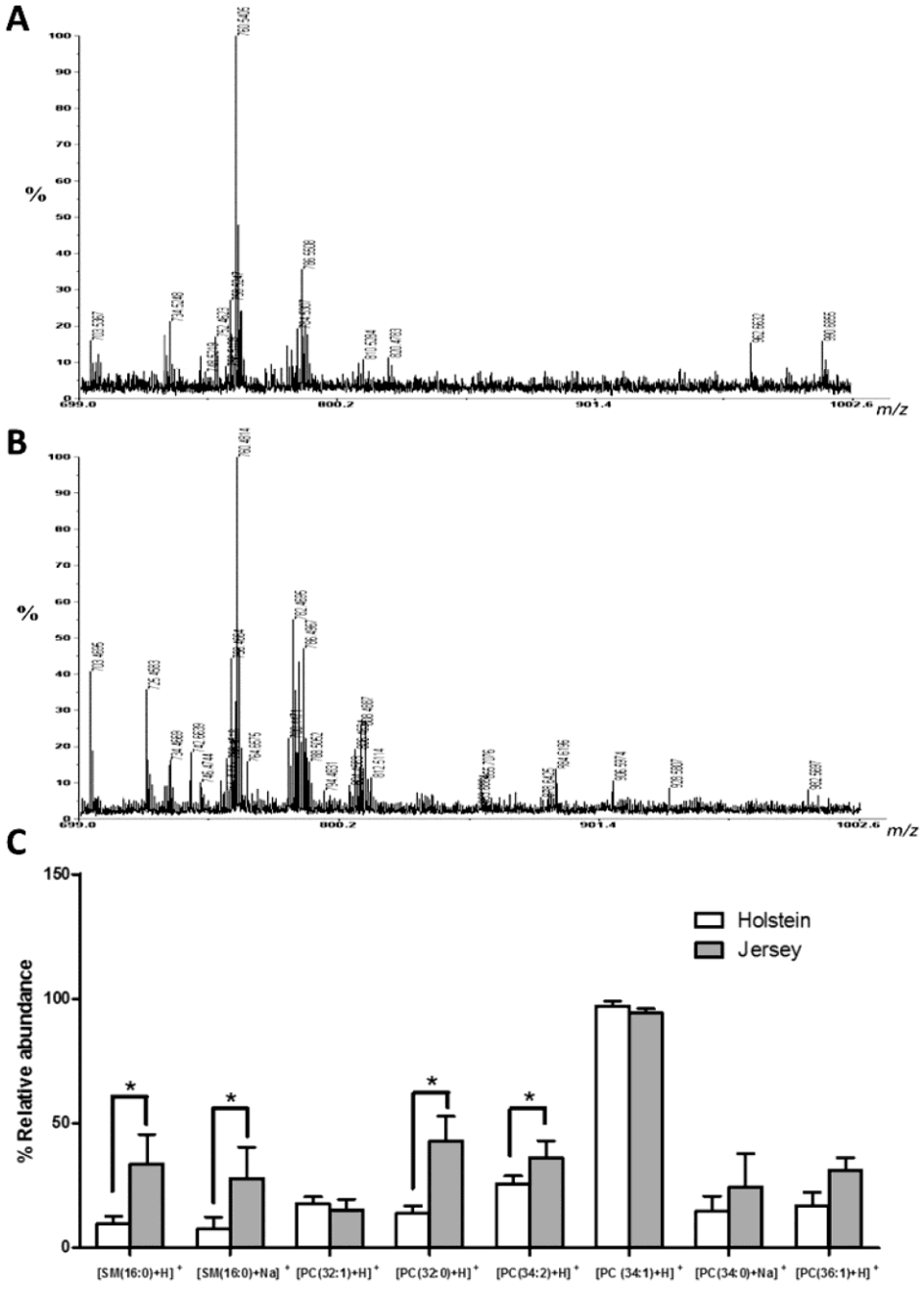
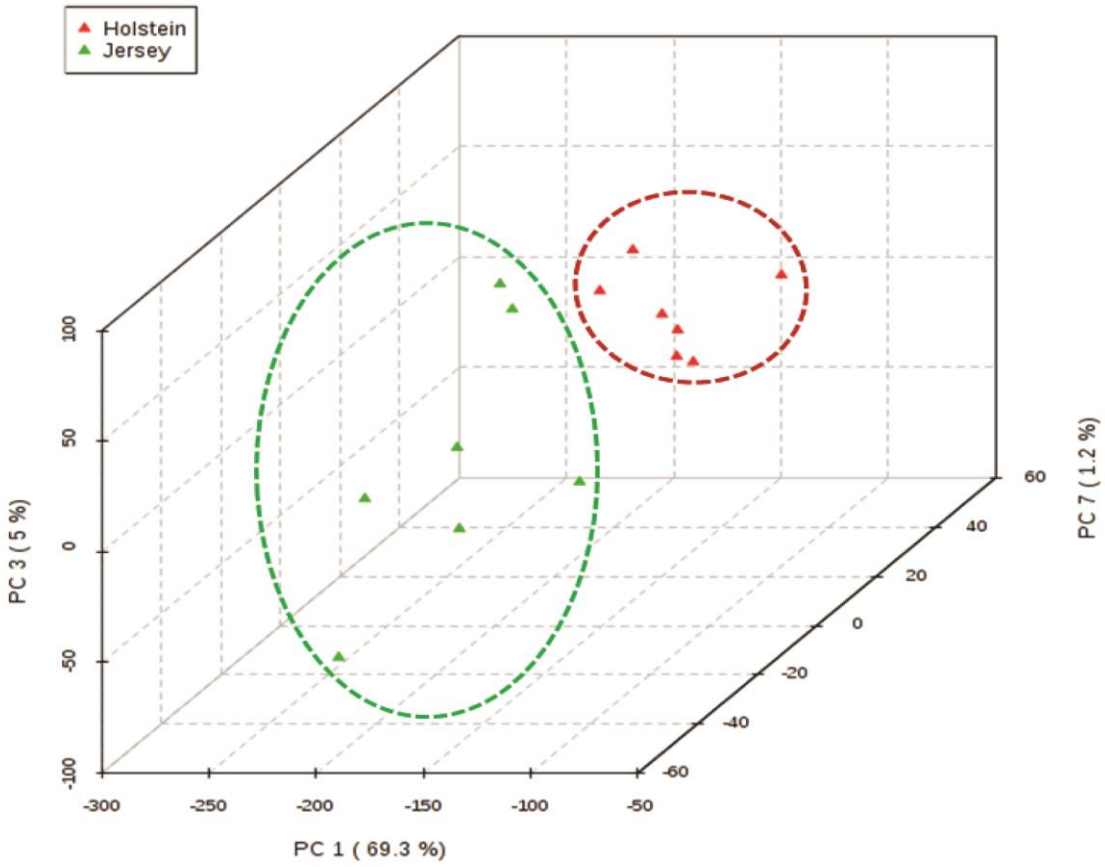


Figure 5. MALDI-MS spectra (positive ion mode) of lipids in Holstein (A) and Jersey (B) embryos. (C) Relative abundance of lipid ions (SM = sphingomyelin, PC = phosphatidylcholine). Values are mean ± SEM. *Significant difference ($P < 0.05$).



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731 **Figure 6.** 3D representation of principal component analysis of the MALDI-MS data for

732 Holstein and Jersey embryo lipid content.

733

734 **Table 1.** Genbank accession, primer sequences, annealing temperatures and product size
735 of candidates used for validation of relative gene expression levels in bovine embryos by
736 quantitative RT-PCR

Symbol	Accession	Primer sequences		Annealing (T°)	Acquisition (T°)	Product size (bp)
		Fw (5'-3')	Rv (5'-3')			
ADIPOR2	NM_001040499	CGCAACTGGGAAGAGAAAAC	CCACCCCTCAGAGGACATAA	57	87	236
LPIN1	NM_001206156	GAGGGGAAGAAACACCACAA	GTCGTCCCAGTTCCACAAGT	57	87	346
LPIN2	XM_592307	AGATCCGAGTCCCACATGGA	CCCGGAAGTGGGTGTTTTCT	57	84	130
ELOVL5	NM_001046597	CACGGTCCTGCATGTGTATC	AAGGTACACGGCCAGATGAC	57	85	264
Mx1	AY_340484	ATGCGTGCTATTGGCTCTTCCTCA	CAAACAGAGCAAGGGAGTTTGGCA	60	85	181
12s	J0_1394	TCGATAAACCCCGATAAACC	TTCGTGCTTGATTCTCTTGG	58	76	186

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739

740 **Table 2.** The most significant phosphatidylcholine (PC) and sphingomyelin (SM) ions
741 identified based on MALDI-MS data obtained from individual bovine oocytes and
742 embryos

m/z	Lipid ion (C atoms: unsaturation)
703.5	[SM (16:0) + H] $^{+}$
725.5	[SM (16:0) + Na] $^{+}$
732.5	[PC (32:1) + H] $^{+}$
734.5	[PC (32:0) + H] $^{+}$
758.6	[PC (34:2) + H] $^{+}$
760.5	[PC (34:1) + H] $^{+}$
782.6	[PC (34:6) + H] $^{+}$, [PC (34:1) + Na] $^{+}$
784.6	[PC (34:0) + Na] $^{+}$
786.6	[PC (36:2) + H] $^{+}$
788.6	[PC (36:1) + H] $^{+}$
802.6	[PC (36:5) + Na] $^{+}$
810.6	[PC (38:4) + H] $^{+}$, [PC (36:1) + Na] $^{+}$

743 Identification is based on the collision induction dissociation database and on earlier
744 studies (Ferreira *et al*., 2010; Sudano *et al*., 2012).

745