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

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Abstract. Some embryos exhibit better survival potential to cryopreservation than others. The cause of such a phenotype is still unclear and may be due to cell damage during cryopreservation, resulting from overaccumulation and composition of lipids. In cattle embryos, *in vitro* culture conditions have been shown to impact the number of lipid droplets within blastomeres. Thus far, the impact of breed on embryonic lipid content has not been studied. In the present 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, with those of *in vivo* Holstein embryos, which have good cryotolerance. Even when housed and fed under the same conditions, Jersey embryos were found to be darker and contain more lipid droplets than 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.

Additional keywords: embryo, lipid droplets, lipid profile, mitochondria.

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Introduction

Over the years, dairy milk production has increased steadily due to several factors, including improved management, nutrition and breeding programs (Lucy 2001). Combined with advances in assisted reproduction technology, such as AI, superovulation, embryo freezing and transfer, conventional breeding has contributed the most to accelerating genetic gain (Bousquet *et al.* 2003). In the field of embryo transfer, there is currently an increase in the demand for frozen rather than fresh embryos (Stroud 2011). Freezing allows storage and transportation of embryos and better management of donors and recipients, and is commercially advantageous because 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 of embryo transfer, including production (*in vivo* or *in vitro*; Crosier *et al.* 2001; Fair *et al.* 2001; Hasler 2001; Rizos *et al.* 2003), composition of the culture media (Yamashita *et al.* 1999; Hasler 2001; Abe *et al.* 2002*b*; 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.* 2002*b*; Abe and Hoshi 2003).

In the dairy industry, cattle breeds differ considerably. The Holstein is recognised for providing the greatest volume of milk, whereas 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 make-up 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 (2004) showed that Jersey embryos frozen in either ethylene glycol or glycerol produced significantly fewer pregnancies than did Holstein embryos. It has been suggested that the lower tolerance of Jersey embryos may 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, 2002*b*; Yamashita *et al.* 1999; Hasler 2001; Reis *et al.* 2003). Changes in mitochondrial structure and function in association with the accumulation of intracellular lipids 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.* 2002*b*; Abe and Hoshi 2003; Rizos *et al.* 2003; Plourde *et al.* 2012). Because mitochondria are not static organelles but vital determinants of normal early embryonic development (Dumollard *et al.* 2007) and are located where ATP must be supplied at high levels (Tarazona *et al.* 2006), these changes should be expected to reduce embryo quality.

Thus far, the effects of the Jersey and Holstein breed on embryonic lipid metabolism have not been documented. We hypothesised that embryonic lipid content differs between the Jersey and Holstein breeds due to their intrinsic differences in lipid management. The present study 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. The present study provides a different perspective to embryonic lipid composition by addressing the need to account for breed-specific differences.

Materials and methods

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

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 subjected to embryo collection at least seven times. Embryos were staged and graded according to the International Embryo Transfer Society (IETS) scores (Robertson and Nelson 1998). Only morula and early blastocysts of Grades 1 and 2 were used in the present study. All breed comparisons were done on samples matched for developmental stage and grade.

Embryos were obtained from L'Alliance Boviteq Inc. (Saint Hyacinthe, Canada). All animals used in this study were handled according to the guidelines provided by the Canadian Council on Animal Care (www.ccac.ca, accessed 1 October 2012). 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 superovulation treatment: Follicles >8 mm in diameter were aspirated on Days 8–12 after oestrus. Administration of FSH (Folltropin-V; Bioniche Animal Health, Belleville, Canada) was started 36 h later (twice daily in doses decreasing from 60 to 20 mg for a total of 400 mg over 4 days). Prostaglandin $F_{2\alpha}$ analogue (Estrumate; Intervet, Kirkland, Canada) was administered in doses of 500 μg with each of the two final FSH injections to initiate luteolysis. Cows in oestrus 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 categorised according to the IETS system. Fresh embryos were needed for some assays, whereas other analyses allowed freezing of the embryos, which were then washed three times in phosphate-buffered saline (PBS), placed in 0.5-mL microtubes in a minimum volume, snap-frozen and stored at −80°C.

Characterisation of lipid droplets and active staining of mitochondria

Mitochondria in fresh blastocyst embryos ($n = 15$ per breed) were stained with 300 nM Chloromethyl-X-rosamine (CMX-Ros), an active dye (Mitotracker Red; Molecular Probes, Eugene, OR, USA) in synthetic oviductal fluid (SOF) for 40 min at 38°C in 5% CO_2. The dye shows strong sensitivity to mitochondrial membrane potential and mitochondrial protein (thiol groups) and exhibits better retention and much more even distribution than other dyes due to high colocalisation 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 used as a negative control to set the background, which was used as a means 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 the addition of CMX-rosamine.

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

Confocal microscopy

Brightfield, confocal and epifluorescence images were acquired using a Nikon TE2000 confocal microscope (Nikon, Mississauga, Canada) with a 60×/1.20 water-immersion objective. Brightfield images of morula embryo morphological phenotype were recorded in grey scale photographs 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, spaced by 0.5 μm, from a first section at the bottom of the 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 × 512 pixel resolution. The excitation and emission wavelengths were as follows: Bodipy493/503, 488 and 515–530 nm, respectively; Mitotracker Red, 555 and 605–675 nm, respectively. All settings were similar for all samples. Mitochondrial activity was recorded as an epifluorescence image of CMX-rosamine dye in grey scale obtained using a Nikon TE2000 microscope equipped with epifluorescence illumination and appropriate filters (G-2a for Mitotracker Red).

Image analysis

The intensity of CMX-rosamine fluorescence was measured using the mean grey scale in ImageJ software (Schneider *et al.* 2012).

Table 1. GenBank Accession no., primer sequences, annealing temperatures and product size of candidates used for validation of relative gene expression levels in bovine embryos by quantitative reverse transcription–polymerase chain reaction

Gene symbol	Accession no.	Primer sequences (5′–3′)	Annealing temperature (°C)	Acquisition temperature (°C)	Product size (bp)
ADIPOR2	NM_001040499	Forward: CGCAACTGGGAAGAGAAAAC Reverse: CCACCCCTCAGAGGACATAA	57	87	236
LPIN1	NM_001206156	Forward: GAGGGGAAGAAACACCACAA Reverse: GTCGTCCCAGTTCCACAAGT	57	87	346
LPIN2	XM_592307	Forward: AGATCCGAGTCCCACATGGA Reverse: CCCGGAAGTGGGTGTTTTCT	57	84	130
ELOVL5	NM_001046597	Forward: CACGGTCCTGCATGTGTATC Reverse: AAGGTACACGGCCAGATGAC	57	85	264
Mx1	AY_340484	Forward: ATGCGTGCTATTGGCTCTTCCTCA Reverse: CAAACAGAGCAAGGGAGTTTGGCA	60	85	181
12s	J0_1394	Forward: TCGATAAACCCCGATAAACC Reverse: TTCGTGCTTGATTCTCTTGG	58	76	186

Results are expressed in arbitrary units (a.u.) 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 plug-in LIPID DROPLET COUNTER of ImageJ software (Schneider *et al.* 2012). 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 fL.

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, Canada) according to the manufacturer's instructions. Genomic DNA was used for mitochondrial (mt) DNA quantification and total RNA was reverse-transcribed and analysed using quantitative reverse transcription–polymerase chain reaction (qRT-PCR).

Quantification of mtDNA

Individual embryos ($n = 10$ per breed) were used to quantify mtDNA using a quantitative PCR (qPCR) method with genomic DNA. The *12S* rRNA gene (GenBank Accession no. J01394) was selected as a mitochondrial target and the *Mx1* gene (GenBank Accession no. AY340484) as a nuclear target (Table 1). The mtDNA and nuclear (n) DNA 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, Laval, Canada) 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 LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) and 5 µL DNA sample. The average DNA concentration of each sample was 0.00335 ng μL^{-1}. The following cycling conditions were used for amplification: initial denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 5 s, 5 s at 58°C (*12S* rRNA) or 60°C (*Mx1*), followed by 72°C for 20 s and 76°C (*12S* rRNA) or 85°C (*Mx1*) for 5 s. The presence of amplicons was verified using melting curve analysis: Following the last amplification cycle, the internal temperature of the LightCycler was increased rapidly to 94°C and then decreased to 72°C for 30 s, followed by a slow increase to 94°C at a rate of 0.1°C s^{-1}, 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.

Differential gene expression in Holstein and Jersey embryos

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

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

Microarray data were pre-processed as described previously (Plourde *et al.* 2012), using Lowess intra-array and quantile inter-array normalisations. Statistically significant variations were detected using Limma (Flexarray, Génome Québec, Montréal, Canada). Differences in gene expression were considered significant for a cut-off adjusted $P < 0.01$ and change of at least 1.2-fold. Pathway analyses and downstream exploitation of gene lists were performed using Ingenuity Pathway Analysis Software Version 8.6 (Ingenuity Systems, Redwood City, CA, USA).

Validation of qRT-PCR

Validation of microarray results was performed using qRT-PCR on additional embryos ($n = 5$ per breed). RNA was reverse transcribed using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) with an oligo-dT to prime the reaction as per the manufacturer's recommendations. Primers were designed for candidates (adiponectin receptor 2 (*ADIPOR2*), Lipin-1 (*LPIN1*), Lipin-2 (*LPIN2*) and ELOVL fatty acid elongase 5 (*ELVOL5*)) using the Primer3 Web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 1 October 2012) and synthesised at IDT (Coralville, IA, USA). The reaction mixture was composed of the LightCycler FastStart DNA Master SYBR Green I kit components (Roche Diagnostics) and real-time measurements were performed in a LightCycler 2.0 apparatus (Roche Diagnostics). Our real-time PCR amplification procedure has been described in detail elsewhere (Gilbert *et al.* 2010). GenBank Accession no., primer sequences, annealing temperatures and product size are given in Table 1.

For quantification, real-time PCR was performed as described previously (Bermejo-Alvarez *et al.* 2010). Each pair of primers was tested for reaction efficiency and the comparative cycle threshold (ΔC_T) method was then used to quantify differences in transcript levels, as described by Schmittgen and Livak (2008). Quantification was normalised ($\Delta\Delta C_T$) to the endogenous control (β-actin to account for cell number). Changes in the relative level of gene expression of the target were calculated as $2^{-\Delta\Delta CT}$.

Analysis of lipid profile by matrix-assisted laser desorption ionisation time–mass spectrometry

Lipid profiles of intact cattle embryos were obtained 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 and stored at −80°C in 0.5-mL microtubes containing 2–4 μL 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, USA) and washed three times in this solution. Each embryo was placed on a single spot on the matrix-assisted laser desorption ionisation (MALDI) target plate. Samples were allowed to dry at room temperature and their location of the samples was 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 time-of-flight (TOF)/TOF instrument (AB Sciex, Concord, Canada) equipped with an Nd : YAG laser operating at 355 nm and 200 Hz. Laser intensity remained fixed for all analyses. External calibration was performed and mass accuracy was better than 50 ppm. The mass spectrometry (MS) spectra were acquired between 700 and 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. The 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 (PCA), which was performed using Pirouette v.3.11 (Infometrix, 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 the present 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 used for comparisons between breeds. Differences were declared significant when $P < 0.05$ (two-tailed). Lipid MS profiles, multivariate and univariate statistical models were used as described previously (Ferreira *et al.* 2010; Sudano *et al.* 2012). A first PCA was performed using Pirouette v.3.11 (Infometrix). Based on the MALDI-MS results, the ions with significant signal intensities over background values 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 Fig. 1. The blastomere cytoplasm was darker in Jersey than 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 methods of Aardema *et al.* (2011). As shown in Fig. 2*a*, *b*, *c*, differences in lipid droplet abundance were observed between the breeds: Jersey embryos had a higher number of droplets and these were of lower average volume than those in the Holstein embryos ($P < 0.05$; Fig. 2*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 Fig. 3*a*, *b*, Holstein and Jersey embryos seem to have a similar mitochondrial distribution, and there was no difference in the mtDNA/nDNA ratio (Fig. 3*d*). However, the

fluorescence intensity was greater in Holstein than Jersey morula (7893 ± 23 a.u.; $P < 0.05$), indicating higher mitochondrial activity in the former (Fig. 3*c*).

Differences in gene expression between Holstein and Jersey breeds

To evaluate differences between embryos of the Holstein and Jersey breeds, a large-scale transcriptome analysis was performed using a microarray. Of the 37 238 transcripts represented on the microarray slide, only 83 protein-coding genes were differentially expressed (>1.2-fold difference; $P < 0.05$), suggesting that the embryos of both breeds are highly similar. The differentially expressed genes were analysed by Ingenuity Pathways Analysis (IPA; http://www.ingenuity.com, accessed 1 September 2012). Among the different biological functions thus identified, we focused on lipid metabolism genes, which may explain the observed differences in lipid content and mitochondrial activity. The analysis revealed that fatty acid release, oleic acid oxidation, palmitic acid uptake and acylglycerol synthesis were the most significant categories of differential lipid metabolism function (data not shown). Validation of the microarray results was performed using qRT-PCR on four genes related to lipid metabolism (*ADIPOR2*, *LPIN1*, *LPIN2*, *ELVOL5*; Fig. 4; Table 1). As observed in the microarray analysis, the expression of these selected genes show a tendency (*P*-value between 0.09 and 0.1) for stronger abundance in Holstein compared to Jersey embryos.

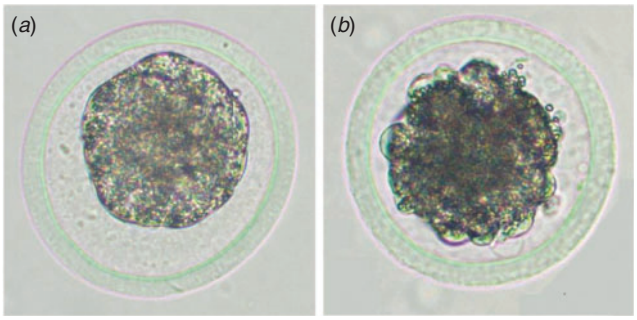

Fig. 1. Morphology of bovine embryos (morula stage) collected *in vivo* 6 days after insemination. (*a*) Holstein, categorised as pale; (*b*) Jersey, categorised as dark. Observed under brightfield microscopy at an original magnification of ×600.

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

In combination with the microarray, a lipid composition analysis was also performed. MS provides a fast and simple means of determining lipid profiles. MALDI-MS can provide a lipid fingerprint of a single intact cattle embryo directly, in particular of phospholipids, such as phosphatidylcholines and

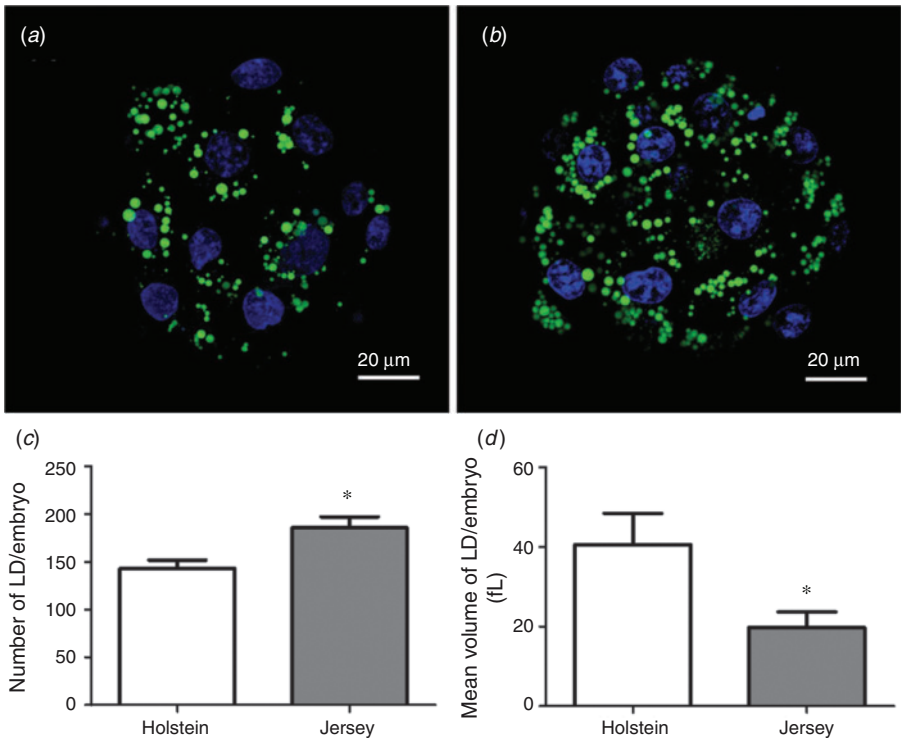

Fig. 2. Orthogonal projections of confocal *z*-stacks representative of lipid droplet content of (*a*) Holstein and (*b*) Jersey morula-stage embryos as revealed by staining with Bodipy 493/503 green dye. DNA is stained with Hoechst blue dye. (*c*) Number of lipid droplets (LD); (*d*) mean lipid droplet volume. Data are the mean ± s.e.m. *$P < 0.05$ compared with the Holstein breed.

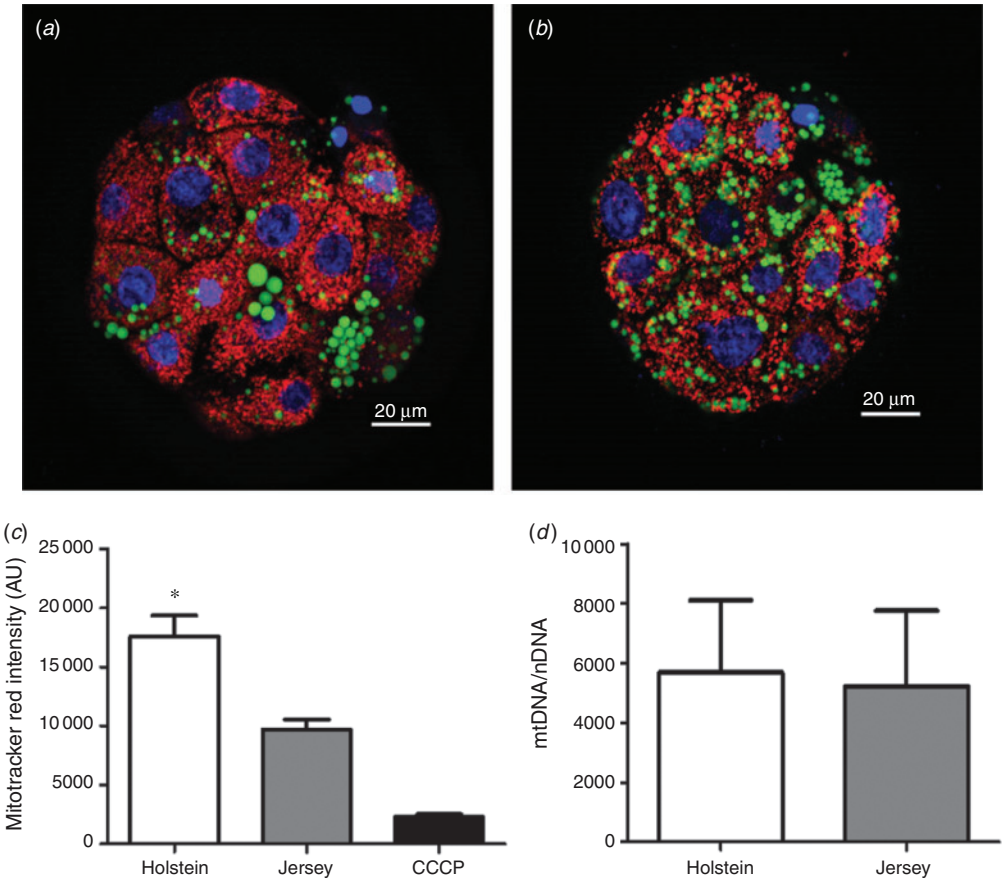

Fig. 3. Confocal microscopic images of (*a*) Holstein and (*b*) Jersey embryos (morula stage) obtained *in vivo* and stained with CMX-rosamine (Mitotracker Red), Bodipy 493/503 (green) and Hoechst blue dye 33342 to show active mitochondria, lipid droplets and nuclear DNA, respectively. Pictures are orthogonal views reconstructed from a confocal image. (*c*) Chloromethyl-X-rosamine (CMX-Ros) fluorescence intensity; carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) was used as a negative control. (*d*) Ratio of mitochondrial to nuclear DNA in single blastocysts. Data are the mean ± s.e.m. $*P < 0.05$ compared with the Jersey breed.

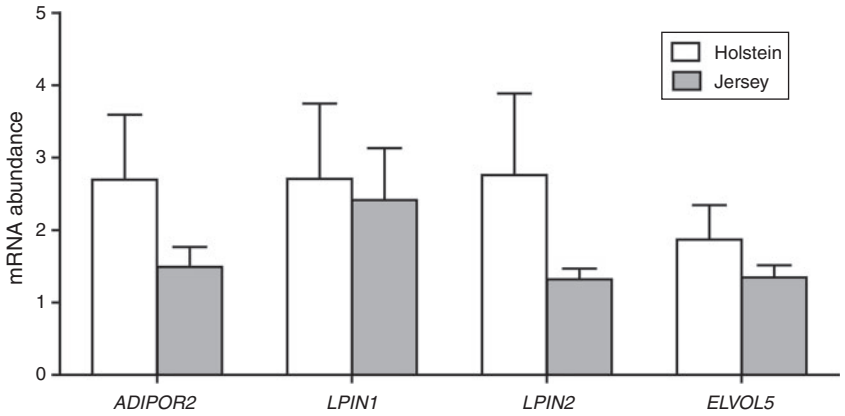

Fig. 4. Quantitative reverse transcription–polymerase chain reaction validation of microarray analysis of transcript levels of genes involved in lipid metabolism, namely adiponectin receptor 2 (*ADIPOR2*), Lipin-1 (*LPIN1*), Lipin-2 (*LPIN2*) and ELOVL fatty acid elongase 5 (*ELVOL5*). Data are the mean ± s.e.m. Expression was normalised against that of endogenous β-actin transcripts to account for cell number.

Table 2. Most significant phosphatidylcholine (PC) and sphingomyelin (SM) ions identified based on matrix-assisted laser desorption ionisation–mass spectrometry data obtained from individual bovine embryos

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

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	$[PC\ (38:4)+H]^+$, $[PC\ (36:1)+Na]^+$

sphingomyelins (Ferreira *et al.* 2010; Sudano *et al.* 2012). The most significant lipids thus identified, based on MALDI-MS, are listed in Table 2. The lipid profiles of embryos of each breed are shown in Fig. 5*a*, *b*. Measurement of lipid ion abundance revealed that protonated sphingomyelin (16 : 0), phosphatidylcholine (32 : 0, 34 : 2) and sodiated sphingomyelin (16 : 0) were significantly higher ($P < 0.05$) in Jersey than Holstein embryos (Fig. 5*c*). PCA of the MALDI-MS data revealed a spatial arrangement in two distinct clusters corresponding to the cattle breeds, with no overlap (Fig. 6).

Discussion

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

The colour of the blastomere cytoplasm is considered an accurate indicator of embryo quality (Lindner and Wright 1983; Thompson *et al.* 1995; Sata *et al.* 1999; Abe *et al.* 2002*b*; Van Soom *et al.* 2003; Guignot 2005) and appears to be a predictor of embryo tolerance of cryopreservation (Yamashita *et al.* 1999; Fair *et al.* 2001; Massip 2001; Abe *et al.* 2002*b*; Van Soom *et al.* 2003; Guignot 2005). However, evaluation of this criterion is subjective, and many factors, including breed, can influence colouration. 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.* (2002) found that Nellore (*Bos indicus*) embryos were 'pale' compared with Holstein embryos. Embryo quality was found to be 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-fertilisation 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.* (2012) reported that the lipid content is higher in Simmental (*Bos taurus*) embryos than in Nellore embryos.

The results of the present study confirmed that the blastomeres of Jersey embryos are darker in colour, mainly due to the abundance of lipid droplets, as suggested by Steel and Hasler (2004). However, average lipid droplet volume was higher in Holstein than 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 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, apparently due to lower mitochondrial activity. This is in agreement with Visintin *et al.* (2002), who reported a stronger inverse relationship between the number of lipid droplets and the number of mitochondria in Holstein embryos compared with Nellore, and with the findings of Abe *et al.* (2002*a*, 2002*b*), who observed fewer mature mitochondria in association with a number of higher lipid droplets in blastomeres with darker cytoplasm in morulas obtained *in vivo* and subsequently classified as embryos of lower quality. In addition, Abe *et al.* (2002*a*, 2002*b*) 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 number of lipid droplets present.

Analysis of gene expression using a microarray did not reveal many differences overall between the two dairy cow breeds. However, the 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

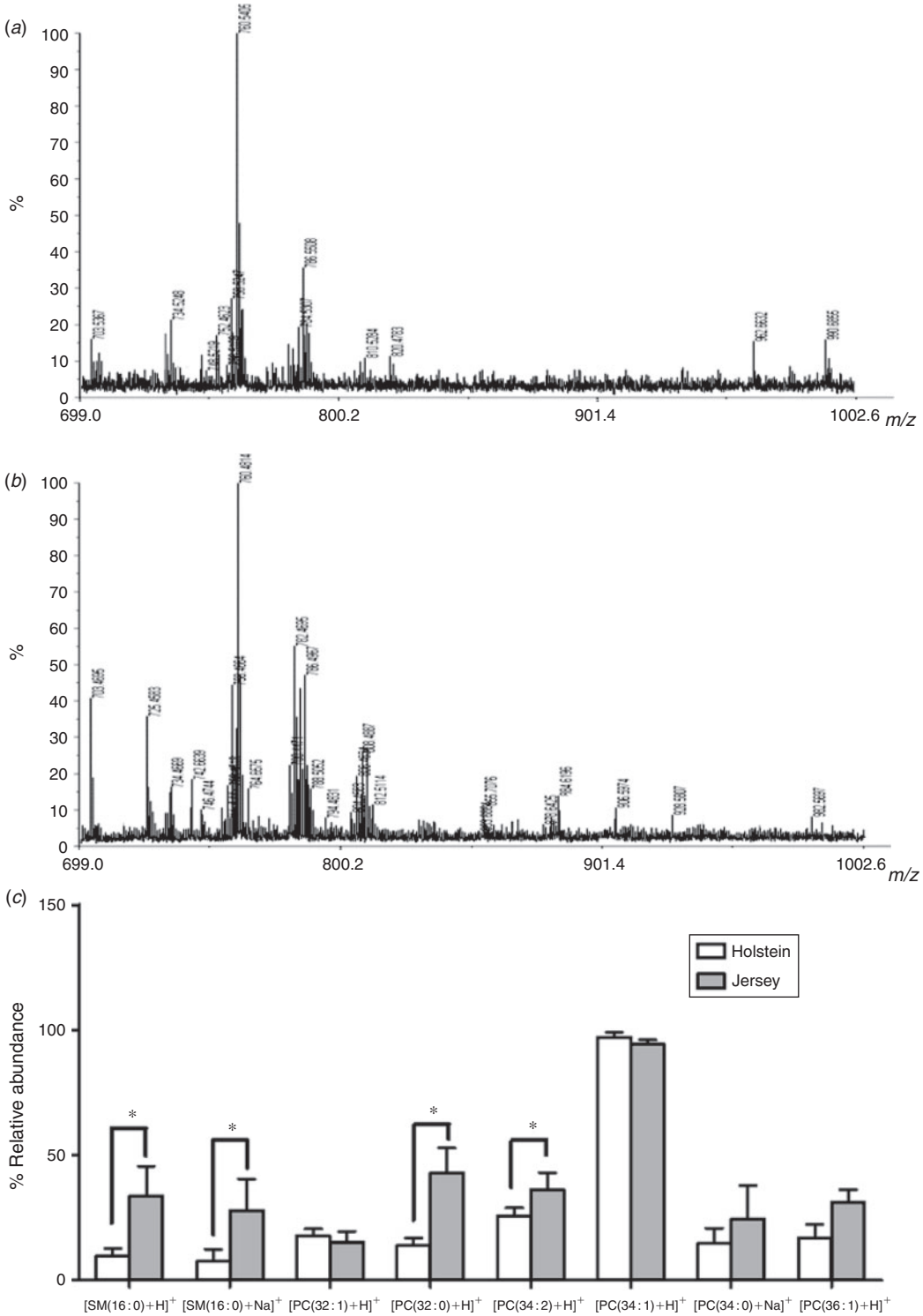

Fig. 5. Matrix-assisted laser desorption ionisation time-of-flight–mass spectrometry spectra (positive ion mode) of lipids in (*a*) Holstein and (*b*) Jersey embryos. (*c*) Relative abundance of lipid ions. SM, sphingomyelin; PC, phosphatidylcholine. Data are the mean ± s.e.m. $*P < 0.05$.

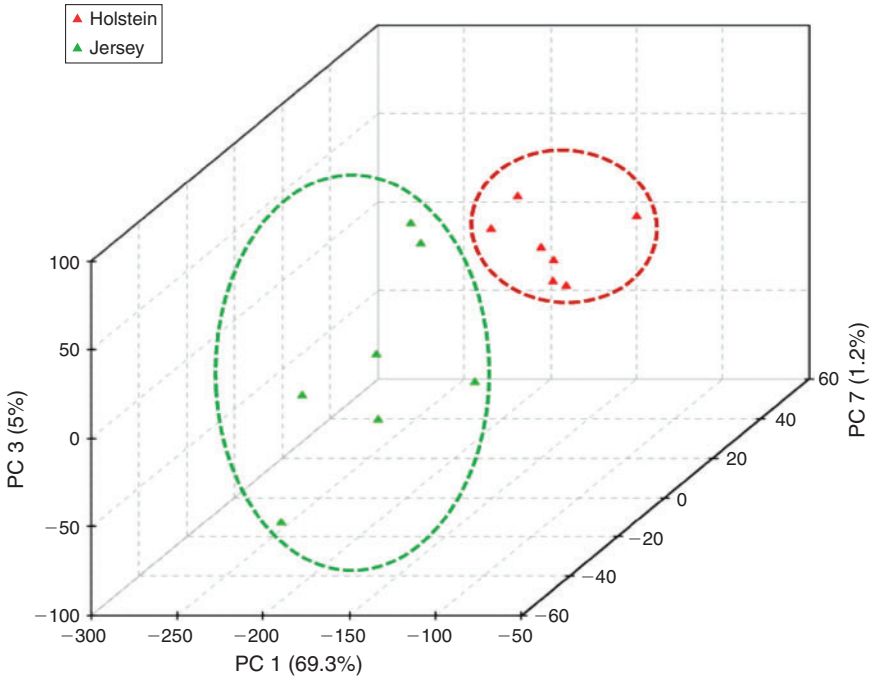

Fig. 6. Three-dimensional representation of principal component analysis of the matrix-assisted laser desorption ionisation time-of-flight–mass spectrometry data for Holstein and Jersey embryo lipid content.

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

It has been reported previously that lipid content plays an important role in determining the characteristics of cell membranes and that modifying their physical properties is crucial for the successful cryopreservation of bovine embryos (Sata *et al.* 1999; Kim *et al.* 2001). Several methods have been developed to evaluate the lipid profile of embryos. However, a major limiting factor is the amount of biological material available for study. Nevertheless, Ferreira *et al.* (2010) and Sudano *et al.* (2012) obtained lipid profiles of embryos with limited quantities of sample using MALDI-MS. As expected, we observed an abundance of positive ions well represented in lipid profiles obtained previously in MALDI-MS studies of *in vivo* bovine embryos (Ferreira *et al.* 2010; Sudano *et al.* 2012). It has been suggested that cow breed influences the lipid profile observed in embryos (Sata *et al.* 1999; Sudano *et al.* 2012). This is consistent with our finding that the lipid profiles of Holstein and Jersey embryos do not overlap.

Elevated numbers of lipid droplets have been associated previously with variable abundance of lipid ions known to vary in association with cow breed (Sudano *et al.* 2012). Although Jersey embryos contained sphingomyelins (16 : 0 $+H^+$ and 16 : 0 $+Na^+$) in abundance, these have been found to have not much impact on embryo cryopreservation (Ferreira *et al.* 2010; Kalo and Roth 2011; Sudano *et al.* 2012). We also noted that Jersey embryos were richer in phosphatidylcholine (32 : 0 $+H^+$ and 34 : 2$+H^+$) identified as palmitic (16 : 0) and linoleic (18 : 2) fatty acids, as described for *Bos taurus* (Sudano *et al.* 2012). It remains unclear how linoleic acid content affects embryo tolerance of cryopreservation. A positive effect of conjugated linoleic acid on cryopreservation of embryos produced *in vitro* has been attributed to a reduction in the number of lipid droplets in cells (Pereira *et al.* 2007). In contrast, Marei *et al.* (2010)

reported a negative effect of linoleic acid on cryopreservation of oocytes matured in culture medium, but this effect is dependent on concentration and is reversible. Some studies 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 acids is critical for the cryopreservation of Holstein and Jersey embryos. Because the Jersey breed is known to produce milk with a higher fat content than Holstein, we hypothesised that the follicle environment of these cows is also lipid enriched. This situation could explain, at least in part, the observed differences in fatty acid composition between Jersey and Holstein embryos. However, this link between follicular environment and embryo composition still needs to be explored. Previously, we treated *in vitro*-produced embryos from a Jersey and Holstein genetic background with L-carnitine in order to reduce embryonic lipid content through increased embryonic metabolism (Baldoceda *et al.* 2016). The results showed an L-carnitine-induced reduction of lipids in both breeds due to increased mitochondrial activity, with milder variations being measured in Jersey embryos. Together, these results show that breed is an important factor to consider when exploring new or improving existing *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 present study focused on the previous observation that Jersey embryos are more sensitive to freezing than Holstein and aimed to contrast these two genetic backgrounds; 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 breed compared with the Holstein 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 the two genetic backgrounds. These results provide clear evidence that, under identical management, breed differences exist at least at in embryonic lipid composition.

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