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Genetic influence on the reduction in bovine embryo lipid content by L-carnitine

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Abstract. The decreased rate of pregnancy obtained in cattle using frozen *in vitro* embryos compared with *in vivo* embryos has been associated with over-accumulation of intracellular lipid, which causes cell damage during cryopreservation. It is believed that the higher lipid content of blastomeres of bovine embryos produced *in vitro* results in darker-coloured cytoplasm, which could be a consequence of impaired mitochondrial function. In this study, L-carnitine was used as a treatment to reduce embryonic lipid content by increasing metabolism in cultured bovine embryos. We have observed previously that *in vivo* embryos of different dairy breeds collected from cows housed and fed under the same conditions differed in lipid content and metabolism. As such, breed effects between Holstein and Jersey were also examined in terms of general appearance, lipid composition, mitochondrial activity and gene expression. Adding L-carnitine to the embryo culture medium reduced the lipid content in both breeds due to increased mitochondrial activity. The response to L-carnitine was weaker in Jersey than in Holstein embryos. Our results thus show that genetics influence the response of bovine embryos to stimulation of mitochondrial metabolism.

Additional keywords: dairy breed, *in vitro*-produced embryo, lipid droplets, lipid profile, mitochondria.

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Introduction

Transfer of embryos produced *in vitro* and preserved by freezing has become routine in dairy production to increase the number of offspring from genetically superior cows (Bousquet *et al.* 1999; Hasler 2006). Despite advances in assisted reproductive technology, these embryos do not tolerate cryopreservation as well as embryos obtained *in vivo* (Hasler 2001; Guignot 2005; Seidel 2006). The resulting lower frequency of pregnancy has been found to be associated mainly with higher cellular lipid levels (Yamashita *et al.* 1999; Abe *et al.* 2002; Seidel 2006). Several groups have reported that producing embryos in serum-containing culture media yields blastomeres with high lipid-droplet contents (Thompson *et al.* 1995; Abe *et al.* 1999; Fair *et al.* 2001). This modifies the embryo lipid profile, which affects tolerance of freezing (Sata *et al.* 1999). Excessive formation of lipid droplets has also been associated with variations in mitochondrial function, which likely affects lipid metabolism (Kruip *et al.* 1983; Dorland *et al.* 1994; Thompson *et al.* 1995; Fair *et al.* 2001; Abe *et al.* 2002; Plourde *et al.* 2012).

This problem has been examined extensively and many possible solutions have been tested in attempts to improve the performance of frozen *in vitro*-produced (IVP) bovine embryos, such as serum-free culture media (Abe *et al.* 2002; Rizos *et al.* 2003), lipid removal (Murakami *et al.* 1998; Diez *et al.* 2001) and supplementation with different fatty acids (Pereira *et al.* 2007; Shehab-El-Deen *et al.* 2009; Aardema *et al.* 2011; Van Hoeck *et al.* 2011). Some positive experimental results have been obtained, but none of these approaches has met with notable success in commercial practice.

Reported in recent studies, another means of reducing intracellular lipid content might be to add L-carnitine (a co-factor of fatty-acid transport into the mitochondrial matrix) to the embryo culture medium (Phongnimitr *et al.* 2013; Takahashi *et al.* 2013). This metabolic regulator could have the dual effects of regulating both lipid levels and accumulation of reactive oxygen species (ROS), thus improving blastocyst development and cryotolerance.

In North America, most studies of bovine embryo transfer have been conducted using the Holstein breed, which is the

most common dairy cow (Hasler 2006). However, important differences between commercial breeds have been observed, of which the consequences for embryo cryotolerance have not yet been studied in any depth. The Jersey breed, valued for its milk fat content, does not provide *in vitro* embryos that respond well to freezing in comparison with the Holstein breed (Steel and Hasler 2004).

The mechanisms underlying the lower frequency of pregnancy in Jersey cows following transfer of cryopreserved IVP embryos are not well understood. We have observed recently that Holstein and Jersey embryos differ somewhat in terms of morphology, lipid profile and molecular characteristics, suggesting that lipid metabolism might be a factor. The goal of the present study was therefore to determine whether or not L-carnitine added to the IVP medium accelerates lipid metabolism and thereby reduces blastomere lipid content. We focussed on the response of phenotype and gene expression levels in Jersey and Holstein embryos.

Materials and methods

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

Animals and embryo production conditions

Non-lactating Holstein ($n = 4$) and Jersey ($n = 4$) cows of 3 years old on average, were fed the same diet and kept under the same conditions at L'Alliance Boviteq Inc., a research farm in Saint-Hyacinthe, Québec, Canada. Embryos were produced at this same location. All animals used in this study were handled following the guidelines provided by the Canadian Council on Animal Care.

Superovulation and oocyte recovery

To initiate a new follicular wave, all follicles larger than 5 mm in diameter were punctured on Days 8–11 after oestrus. Administration of follicle-stimulating hormone (Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada) was begun 36 h after follicular removal. FSH was injected twice a day in doses of 40 mg for a total of 240 mg (six injections). The ultrasound-based pick-up procedure was performed 48 h after the final injection using an 18-gauge needle and COOK aspiration device (COOK Medical, Bloomington, IN, USA). Cumulus–oocyte complexes (COC) thus recovered were submitted to *in vitro* maturation.

In vitro *maturation of oocytes*

COCs were twice washed thoroughly in HEPES-buffered Tyrode's medium containing 10% bovine serum, 200 μM pyruvate and 50 μg mL^{-1} gentamycin to ensure total removal of follicular liquid. Healthy COCs were then placed in maturation medium (groups of 10 per 50-μL droplet) under filtered mineral oil (9 mL) for 24 h at 38.5°C in a humidified 5% CO_2, 20% O_2 atmosphere. The maturation medium contained TCM199 (Invitrogen, Burlington, ON, Canada), 10% fetal calf serum (Sterile Fetal Bovine Serum for Cell Culture; Medicorp, Montreal, QC, Canada), 200 μM pyruvate, 50 μg mL^{-1} gentamycin and 0.1 μg mL^{-1} FSH (Gonal-f; Serono Canada Inc., Mississauga, ON, Canada).

In vitro *fertilisation*

Washed and matured COCs were placed in groups of five per droplet (50 μL) of modified Tyrode's lactate medium (containing 0.6% (w/v) bovine serum albumin (BSA; Fraction V) and 200 μM gentamycin) under filtered mineral oil. Two μL of a solution containing 1 mM hypotaurine, 2 mM penicillamine and 250 mM epinephrine were then added to each droplet. *In vitro* fertilisation with frozen semen (pooled ejaculate) of the Jersey or Holstein breed (provided by L'Alliance Boviteq) was performed without delay. The semen was thawed at 37°C in a water bath, laid on a discontinuous Percoll gradient (2 mL of 45% Percoll over 2 mL of 90% Percoll) and centrifuged at 700*g* for 30 min at room temperature. The supernatant was discarded and the pellet was re-suspended in modified Tyrode's lactate medium such that 50 000 spermatozoa (based on count using a haemocytometer) were used to fertilise each group of five COCs.

In vitro *culture of embryos*

Presumptive zygotes were stripped of cumulus cells and spermatozoa by gentle pipetting in pre-incubated synthetic oviduct fluid then allocated randomly to two groups for culture in synthetic oviduct fluid (SOF) either with 0.5 mM L-carnitine (the Treated or +LC group) or without (the Control or –LC group). The SOF medium was a standard culture medium containing amino acids and 0.4% fatty-acid-free BSA (ICP-Bio, Auckland, New Zealand). L-carnitine was added and dissolved directly in the medium along with the other compounds that are added to the base stock solution. Ten zygotes were placed in a single droplet (10 μL) under filtered mineral oil. The culture dishes were incubated at 38.5°C in a humidified atmosphere containing 6.5% CO_2, 5% O_2 and 88.5% N_2. Ten embryos were transferred to each fresh droplet (10 μL) at 72 h and 120 h (20 μL) after fertilisation to prevent ammonia intoxication (from amino-acid metabolism) and nutrient depletion. Embryos remained under these conditions until Day 6, when morulas and early blastocysts were categorised according to the International Embryo Transfer Society (IETS) system for comparison purposes. Samples were produced using seven independent *in vitro* production runs. The COCs from different donor cows were pooled within each breed and randomly allocated to treatment or control groups. Morula-stage embryos were used for lipid quantification and mitochondrial activity measurement while blastocysts were used for mitochondrial DNA and RNA analyses.

Mitochondrial and lipid-droplet staining

Mitochondria in morulas ($n = 8$ per group) were stained with the active dye CMX-rosamine (Mitotracker Red; Molecular Probes, Eugene, OR, USA) at a final concentration of 300 nM in SOF for 40 min at 38°C in 5% CO_2. This dye shows strong sensitivity to the mitochondrial membrane potential and affinity for mitochondrial protein (thiol groups) and exhibits better retention in the organelle compared with other dyes, due to high co-localisation with cytochrome C oxidase (Poot *et al.* 1996). Embryos selected randomly were incubated for 15 min with 100 nM carbonyl cyanide m-chlorophenylhydrazone (CCCP, which uncouples mitochondrial membrane potential) before adding the dye, in order to

provide a negative control. The fluorescence excitation wavelength was 594 nm and emission was read at 608 nm. Following staining with CMX-rosamine, the embryos were immersed for 10 min in SOF containing 3 μg mL^{-1} 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) in order to stain cytoplasmic lipid droplets. To label nuclei, embryos were incubated for 10 min at room temperature in SOF containing 1 μg mL^{-1} of Hoechst blue dye 33342. Non-fixed specimens were washed three times in SOF and mounted on the microscope slide in the same medium using 120-μm thick spacers.

Confocal microscopy

Bright-field, confocal and epifluorescence images were acquired using a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada) with a 60× 1.20 water-immersion objective. Bright-field images of morula embryo morphological phenotype were recorded in grey-scale photos taken 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, starting from a first section at the bottom of embryo next to the coverslip. The 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 at a resolution of 512 × 512 pixels. The respective excitation and emission wavelengths were as follows: Bodipy (488 nm, 515–530 nm) and Mitotracker Red (555 nm, 605–675 nm). All the settings were the same for all samples. Mitochondrial activity was recorded as an epifluorescence image of CMX-rosamine dye in grey scale and carried out using a Nikon TE2000 microscope equipped with epifluorescence illumination and appropriate filters, which were G-2a for Mitotracker Red. Digital images of stained embryos were viewed with the Plan-Apochromat 40× lens (NA = 1.2) at 10% utilisable laser intensity (maximum power, 1.2 W; output, 25% of the maximum tube current) and a main dichroic beam splitter (HFT, 458/514 nm). Images of orthogonal projections consisting of 21 slices (1 μm each) were acquired as 'lambda stacks' using the Lambda Mode scanning procedure at a resolution of 1024 × 1024 pixels. Lambda stacks were recorded at a specific wavelength for each dye. The microscope settings and the lambda mode scanning procedure were the same for all collected lambda stacks.

Image analysis

CMX-rosamine fluorescence intensity was measured using the mean grey scale in IMAGE J software (National Institute of Health (NIH), Maryland, USA; Schneider *et al.* 2012). Results are expressed in arbitrary units (AU) as the mean fluorescence intensity of all samples within a group. Measurements of the lipid-droplet number and volume in each optical section were obtained using the ImageJ software Lipid Droplet Counter plugin (Abramoff *et al.* 2004). The minimal droplet size threshold was set at five 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).

Total DNA and RNA isolation

For differential gene expression analyses, total RNA from control (−LC) and treated (+LC) samples was extracted from single blastocyst ($n = 4$ for each group) and purified using a PicoPure RNA kit (Molecular Devices, Downingtown, PA, USA) according the manufacturer's instructions. DNA was digested using DNase I from Qiagen (Mississauga, ON, Canada). RNA was eluted in 11 μL of elution buffer and 1 μL was used to measure the quality and concentration of extracted RNA using a 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) with the RNA PicoLab Chip (Agilent Technologies). Only RNA of very good quality (RNA Intergrity Number over 8) was used for the amplification.

Mitochondrial DNA quantification and quantitative reverse transcription polymerase chain reaction (qRT-PCR) validation were performed using individual blastocysts ($n = 4$ per group). Simultaneous extraction of total genomic DNA and RNA were done using the AllPrep DNA/RNA Micro Kit (Qiagen) following the manufacturer's instructions. The DNA and RNA of each blastocyst were eluted in 30 μL and 10 μL volumes, respectively. The DNA extract was used for mitochondrial DNA quantification and total RNA was reverse transcribed and used for qRT–PCR.

Quantification of mitochondrial DNA

Embryo mitochondrial DNA (mtDNA) was quantified using a quantitative PCR (qPCR) method with genomic DNA. The *12S rRNA* gene (GenBank accession number J01394) was selected as a mitochondrial target and the *Mx1* gene (GenBank accession number AY340484) as a nuclear target (Table 1). The ratio of mitochondrial to nuclear DNA (mtDNA/nDNA) was used to calculate the relative concentration of mitochondrial DNA in each individual embryo. The LightCycler 2.0 (Roche Diagnostics, Laval, QC, 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 of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) and 5 μL of DNA sample. 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 s, 58°C (12S rRNA) or 60°C (Mx1) for 5 s 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. Quantification of mitochondrial and nuclear DNA copy numbers was based on a standard curve made from a serial dilution of a PCR amplicon of the targets.

Differential gene expression in Holstein and Jersey embryos

Purified RNA (10 μL left after the bioanalyser measurement) was then amplified in two rounds using the RiboAmp HSPlus RNA Amplification Kit (Life Science, Foster City, CA, USA) with T7 RNA. The amplicon concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Antisense RNA labelled with Cy3 or Cy5 using the Universal Linkage System (ULS) kit (Kreatech Diagnostic, Amsterdam, Netherlands) was hybridised for 17 h at 65°C on Agilent-manufactured EmbryoGENE slides in aliquots of 825 ng (Robert *et al.* 2011) in a two-colour dye-swap design.

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

Gene symbol	Accession no.	Primer sequences		Annealing T (°C)	Acquisition T (°C)	Product size (bp)
		Forward (5′–3′)	Reverse (5′–3′)			
ADIPOR2	NM_001040499	CGCAACTGGGAAGAGAAAAC	CCACCCCTCAGAGGACATAA	57	87	236
Adenosine triphosphate5D	NM_176670	CTAGTTGTGGTCCACGCTGA	ACTCCAGAGCCTTCACCAAG	57	85	256
CPT2	NM_001045889	TCCTGGATCAAGATGGGAAC	GTGGGACAGGTGGACAAAGT	57	84	254
ACOT4	NM_001098941	GGCCTCCTAGACATTGTGGA	ACATCACGGGTTTGTCCAAT	57	81	289
FADS2	NM_001083444	ACCTGCCTTACAACCACCAG	TGTGACCCACACAAACCAGT	57	86	248
Mx1	AY_340484	ATGCGTGCTATTGGCTCTTCCTCA	CAAACAGAGCAAGGGAGTTTGGCA	60	85	181
12s	J0_1394	TCGATAAACCCCGATAAACC	TTCGTGCTTGATTCTCTTGG	58	76	186

A simple direct comparison between control (−LC) and treated (+LC) embryos from each breed was performed. Microarray slides were then washed and scanned with the PowerScanner (Tecan, Männedorf, Switzerland) and analysed with Array-Pro Analyser software (MediaCybernetics, Bethesda, MD, USA).

Microarray data were pre-processed as described in previous studies (Plourde *et al.* 2012). Microarray raw data are available at Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) under accession number GSE62595. Briefly, data intensity files were analysed by FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray, accessed 10 June 2014), where raw data corrected by background subtraction were pre-processed using the Lowess intra-array normalisation and Quantile inter-array normalisation. Statistically significant variations were detected using Limma algorithm (FlexArray, based on the limma package in Bioconductor; Smyth 2004). Differences in gene expression were considered to be significant when at least 1.5 and the cut-off adjusted P value was <0.01. Pathway analyses and downstream exploitation of gene lists were performed using Ingenuity Pathway Analysis Software Version 8.6 (Ingenuity Systems Inc., Redwood City, CA, USA).

Validation of gene pathways by quantitative RT-PCR

Ten μL of total RNA obtained from the AllPrep DNA/RNA Micro Kit (Qiagen) were 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 in a final reaction volume of 20 μL. Primers of candidates (adiponectin receptor 2 (ADIPOR2), ATP synthase H^+ transporting mitochondrial F1 complex delta subunit (ATP5D), carnitine palmitoyltransferase 2 (CPT2), acyl-coenzyme A thioesterase 4 (ACOT4) and fatty acid desaturase 2 (FADS2)) were designed using the Primer3 Web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 10 June 2014) and synthetised at IDT (Coralville, IA, USA). The reaction was performed using 2 μL of cDNA and the LightCycler FastStart DNA Master SYBR Green I kit components (Roche Diagnostics). Real-time measurements were performed in a LightCycler 2.0 apparatus (Roche Diagnostics). GenBank accession number, primer sequences, annealing temperatures and product size are shown in Table 1.

The reaction conditions have been described previously (Bermejo-Alvarez *et al.* 2010). Each pair of primers was tested to achieve efficiencies close to 1 and the comparative cycle threshold ($^{\Delta\Delta}$CT) method was then used to quantify expression levels (Schmittgen and Livak 2008). Quantification was normalised relative to the level of β-actin expression (endogenous control). The CT of β-actin was subtracted from the CT of the gene to obtain $^{\Delta}$CT. For the calculation of $^{\Delta\Delta}$CT, the highest sample $^{\Delta}$CT value (i.e. from the sample with the lowest target expression) was subtracted from all other $^{\Delta}$CT values. The change in the relative level of gene expression of the target was calculated as $2^{-\Delta\Delta CT}$.

Lipid-profile analyses by matrix-assisted laser desorption–ionisation mass spectroscopy (MALDI-MS)

The lipid profile of intact embryos was analysed using a mass spectroscopy (matrix-assisted laser desorption–ionisation or MALDI) procedure described previously (González-Serrano *et al.* 2013) with some modifications. Briefly, each biological replicate was composed of three morula-stage embryos. Analysis was done on four or five biological replicates per group. Embryos were first washed three times in phosphate-buffered saline (PBS) solution and then stored in 0.5-mL microtubes containing 2–4 μL of PBS at −80°C. Samples were thawed in 100 μL of 50% (v/v) methanol (high-performance liquid chromatography (HPLC) grade; Fisher Scientific, Fair Lawn, NJ, USA) in ultrapure water (Millipore, Billerica, MA, USA) and washed three times in the same solution. Each biological replicate was spotted on a single sample location on the spectrometer probe surface and allowed to dry at room temperature. The matrix, 1 μL of 1.0 M 2,5-dihydroxybenzoic acid diluted in methanol, was then spotted onto each sample location and the spots were allowed to dry at room temperature.

Mass spectra were recorded in reflector mode (positive ions) using an AB SCIEX 4800 MALDI TOF/TOF TM instrument (AB Sciex, Concord, ON, Canada) equipped with a neodymium-doped yttrium aluminium garnet (Nd : YAG) laser operating at 355 nm and 200 Hz. Laser intensity remained constant for all analyses. External calibration was performed and mass accuracy was better than 50 ppm. MS spectra were acquired between 700–1000 Da. The spots received 10 V and 60–90 s laser shots, until

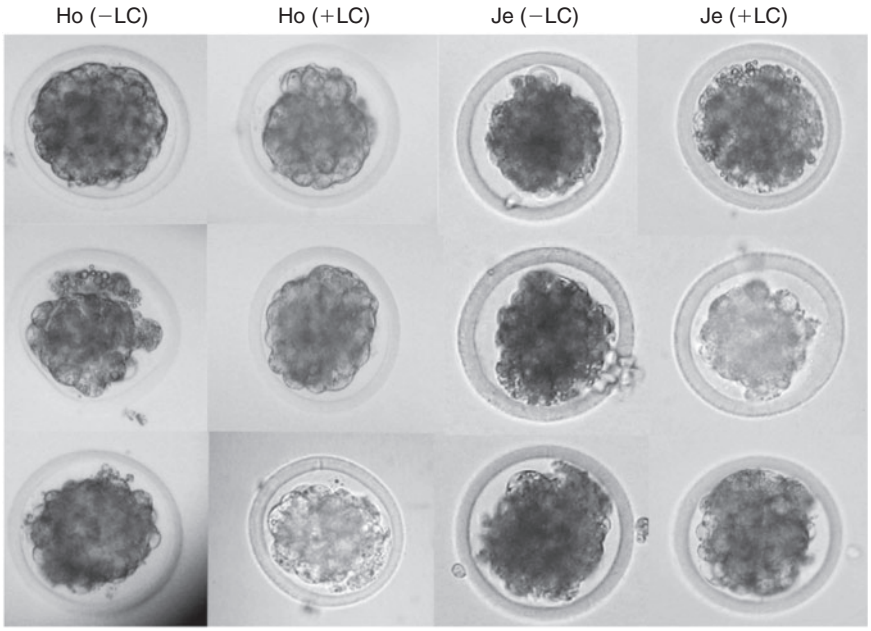

Fig. 1. Phase-contrast images of morula-stage bovine embryos produced *in vitro*. Ho, Holstein; Je, Jersey; −LC, no L-carnitine added; +LC, culture medium enriched with L-carnitine.

the signal from that location disappeared due to ablation of the sample. MALDI-MS databased on collision induction dissociation (CID) were acquired 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 mass-to-charge ratio (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 Version 3.11 (Infometrix Inc., Woodinville, WA, USA). Ion fragmentation patterns obtained in previous studies (Ferreira *et al.* 2010, 2012; Sudano *et al.* 2012) were used to identify the lipids.

Statistical analyses

For the lipid-droplet mean volume and number, data were analysed using the ANOVA procedure in Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA). For RNA abundance values, one-way ANOVA was done in combination with Tukey's multiple-comparisons test. Differences between groups were declared significant when $P < 0.05$. As described previously for lipid mass spectra (Ferreira *et al.* 2010; Sudano *et al.* 2012), multivariate and univariate statistical models were used. A first principal component analysis (PCA) was performed using Pirouette Version 3.11 (Infometrix Inc.) and the MetaboAnalyst website (www.metaboanalyst.ca, accessed 10 June 2014; Xia *et al.* 2009). The relevant ions for group differentiation indicated by the PCA score plot were selected for further univariate analysis using Student's *t*-test in order to confirm their significance as indicated by *P* value.

Results

Impact of L-carnitine on embryo phenotype and lipid content

The overall appearance of the Holstein and Jersey control and L-carnitine-treated morula-stage embryos obtained *in vitro* is shown in Fig. 1. The cytoplasm of blastomeres in the treated group (+LC) appears pale compared to the control group (−LC) regardless of the breed. Some embryos responded more profoundly to the treatment than others as some embryos became very pale. The underlying mechanism for this variability in the reaction to L-carnitine is unknown.

Considered as the storage reservoir of triacylglycerol and cholesterol esters, lipid droplets in embryos were revealed and quantified using a neutral lipid stain (Bodipy) according to Aardema *et al.* (2011). As shown in Fig. 2, differences in lipid-droplet content are apparent between the control (−LC) and treated (+LC) groups for both breeds. The number of droplets was lower in the treated groups even when the breeds were considered together ($P < 0.05$, Fig. 3*a*), while the average volume tended to be lower (Fig. 3*b*) for the comparisons within breed.

Effects of L-carnitine on mitochondrial activity

Based on Mitotracker dye intensity (Poot *et al.* 1996), changes in the intensity of active mitochondria (in red) can be observed in bovine embryos. As expected, the fluorescence intensity was greater in the treated (+LC) group than in the control (−LC) group in both breeds (Holstein, 9491 ± 24 AU; Jersey, 7102 ± 29 AU; $P < 0.05$, Fig. 4*a*). This result indicates that the mitochondria were more active, since the difference between the experimental treatment (+LC) and the control (−LC) group did not affect the ratio of mitochondrial-to-nuclear DNA in either breed (Fig. 4*b*).

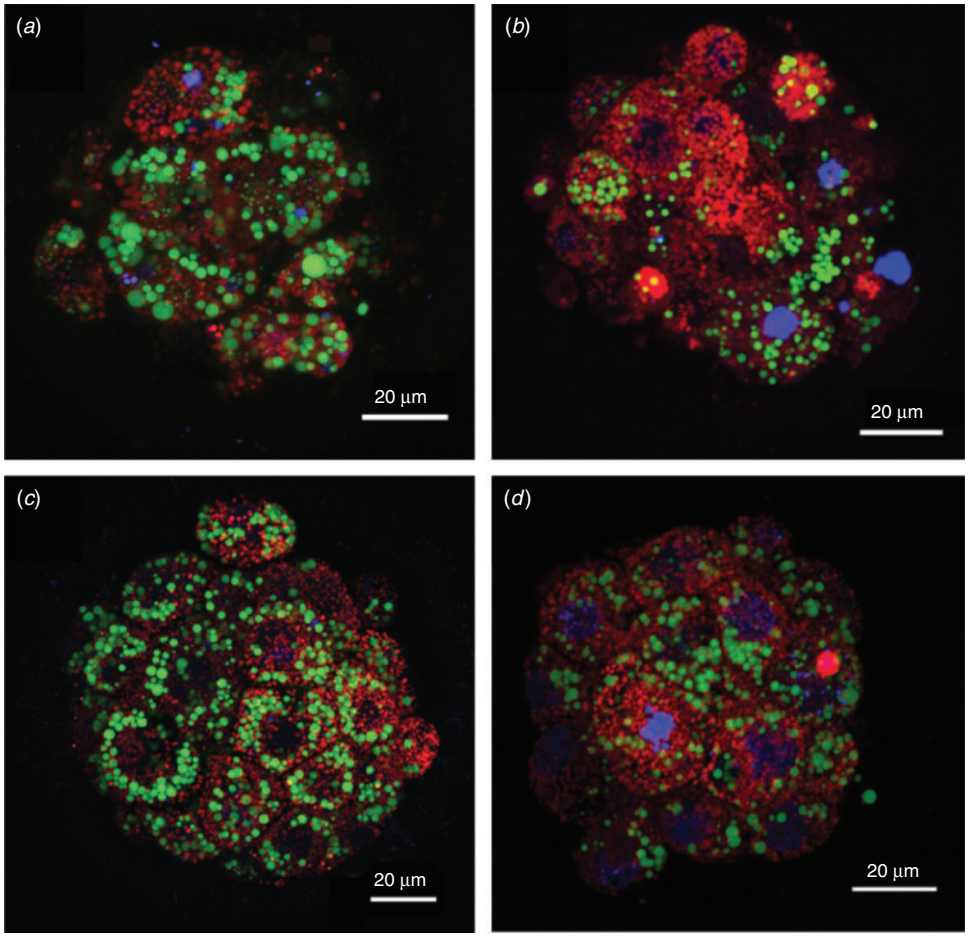

Fig. 2. Three-dimensional orthogonal projection of confocal images of active mitochondria labelled with Mitotracker Red (red), lipid droplets labelled with Bodipy 493/503 (green) and DNA labelled with Hoechst dye (blue) in morula-stage bovine embryos produced *in vitro*. (*a*) Holstein, no L-carnitine (−LC) added, (*b*) Holstein, culture medium enriched with L-carnitine (+LC), (*c*) Jersey, no L-carnitine (−LC) added, (*d*) Jersey, culture medium enriched with L-carnitine (+LC).

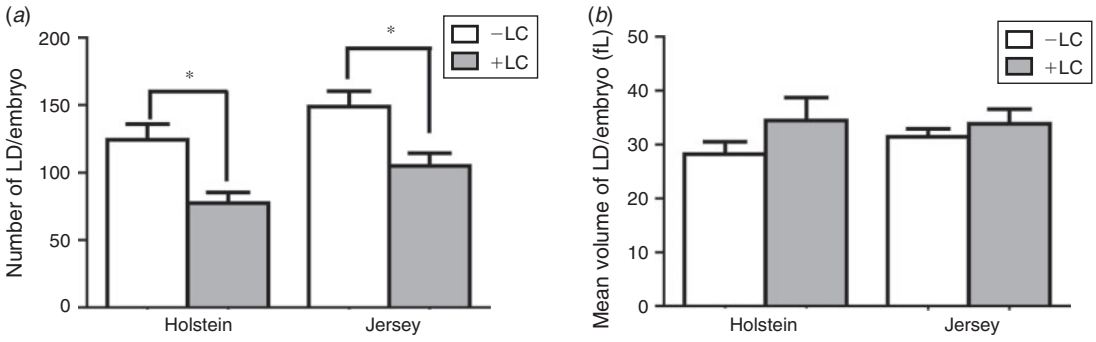

Fig. 3. Lipid droplets (LD) in morula-stage bovine embryos produced *in vitro*. (*a*) Number, (*b*) mean volume in femtolitres. −LC, no L-carnitine added to culture medium; +LC, L-carnitine added to culture medium. Bars represent mean ± s.e.m. $*P < 0.05$.

Gene expression profile

A large-scale transcriptomic comparison of the control (–LC) and treated (+LC) groups of Holstein and Jersey embryos at the blastocyst stage was obtained using a microarray. Based on statistical analysis, 646 transcripts were more strongly expressed in the Holstein breed, while 177 targets were more strongly expressed in Jersey embryos. The corresponding molecular and cellular functions were cell cycle, cellular movement,

carbohydrate, lipid and small molecule metabolism in the Holstein breed and cell-to-cell signalling and interaction, cellular compromise, cellular function and maintenance, cellular development and cellular growth and proliferation in the Jersey breed. We then focussed on the known effect of L-carnitine relevant to carbohydrate and lipid metabolism to explain the differences in lipid levels and mitochondrial activity observed between the two treatments in Holstein embryos. Five genes (*ADIPOR2*, *ATP5D*, *CPT2*, *ACOT4*, *FADS2*) related to carbohydrate and lipid metabolism (Table 1) were selected. The results show a tendency for stronger expression of *CPT2* and *FADS2* ($P < 0.1$) in Holstein embryos subjected to L-carnitine treatment (Fig. 5).

Effect of L-carnitine on the lipid profiles of Holstein and Jersey embryos obtained in vitro

Matrix-assisted laser desorption–ionisation mass spectrometry (MALDI-MS) can provide a lipid fingerprint of a single intact bovine embryo, in particular the profile of phospholipids such as phosphatidylcholines and sphingomyelins (Ferreira *et al.* 2010, 2012; Apparicio *et al.* 2012; Sudano *et al.* 2012; Tata *et al.* 2013). Based on principal component analysis (PCA), the most significant lipids were identified (Table 2). A representative lipid profile of each group is shown in Fig. 6. The interactions between cattle breed and L-carnitine supplementation affected the lipid profiles of the four experimental groups in different ways. Phosphatidylcholine 34 : 6 protonated or 34 : 1 Na^+ ion structure (m/z 782.6) were significantly less abundant ($P < 0.05$) in treated (+LC) than control (–LC) embryos of the Holstein breed (Fig. 7). This phosphatidylcholine was also significantly less abundant in Holstein than in Jersey embryos subjected to the L-carnitine treatment (Fig. 7). The treatment also tended to decrease the relative abundance of 16 : 0 sphingomyelin (protonated ions m/z 703.5 and $+Na^+$ m/z 725.5) in the case of Holstein embryos, while doing the opposite in the case of Jersey embryos. Untreated embryos tended ($P > 0.05$) to contain less 32 : 1 phosphatidylcholine (protonated ion m/z 732.5) in both breeds (Fig. 7). Phosphatidylcholines of 34 : 2 and 32 : 0 structures (protonated ions m/z 758.6 and 734.5, respectively) were found at similar relative abundance ($P > 0.05$)

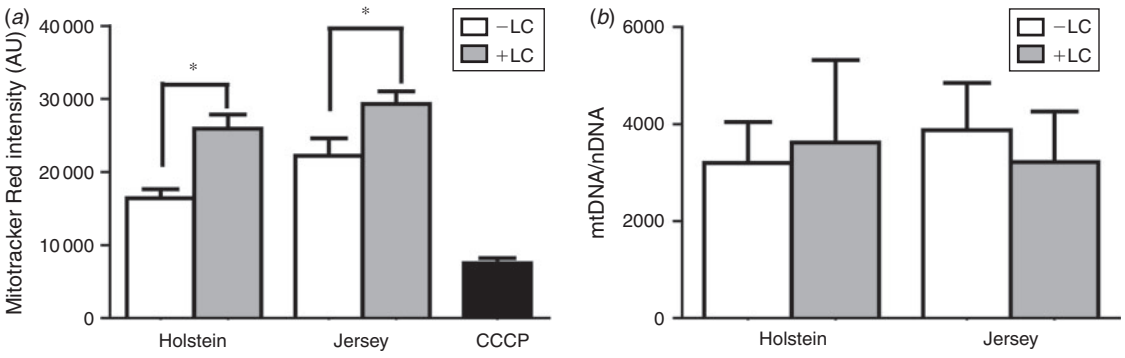

Fig. 4. (*a*) Fluorescence intensity (in arbitrary units, AU) of Mitotracker Red and (*b*) ratio of mitochondrial (mt) to nuclear (n) DNA in bovine embryos produced in culture media with (+LC) or without (−LC) added L-carnitine. Medium containing carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a negative control. Bars represent mean ± s.e.m. $*P < 0.05$.

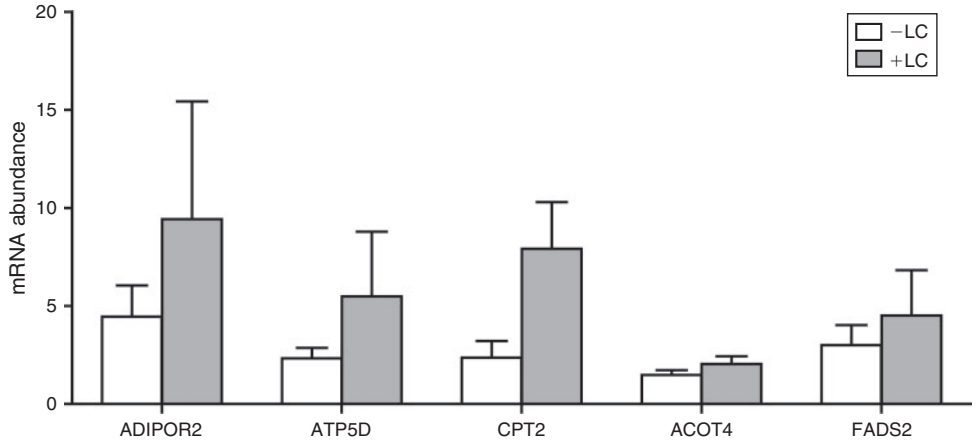

Fig. 5. Validation by quantitative RT-PCR of microarray results for gene expression levels in Holstein blastocyst embryos produced *in vitro*, using five genes involved in carbohydrate and lipid metabolism. −LC, no L-carnitine added to culture medium; +LC, L-carnitine added to culture medium. Bars represent mean ± s.e.m. Quantities are normalised relative to endogenous β-actin transcripts.

Table 2. The most significant phosphatidylcholine (PC) and sphingomyelin (SM) ions identified based on MALDI-MS 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	$[PC (38:4) +H]^+$, $[PC (36:1) +Na]^+$

among the four groups (Fig. 7). Principal component analysis revealed two distinct clusters corresponding to the control (–LC) and treated (+LC) Holstein embryos, while the control (–LC) and treated (+LC) Jersey embryos overlapped with the Holstein control group and with all treatments, respectively (Fig. 8).

Discussion

In this study, L-carnitine had the effect of changing the colour of blastomeres, making both Holstein and Jersey embryos appear paler than embryos produced in a standard *in vitro* medium. L-carnitine thus could be used to select embryos with a higher tolerance to cryopreservation, since it has been reported in several studies that pale colour of the blastomere cytoplasm is a reliable indicator of embryos with superior tolerance to cryopreservation (Sata *et al.* 1999; Yamashita *et al.* 1999; Hasler 2001; Van Soom *et al.* 2003). However, the effect of L-carnitine on colour was lesser in Jersey than in Holstein embryos. It has been reported that the coloration of the blastomere cytoplasm is due to the number of lipid droplets and varies among cattle

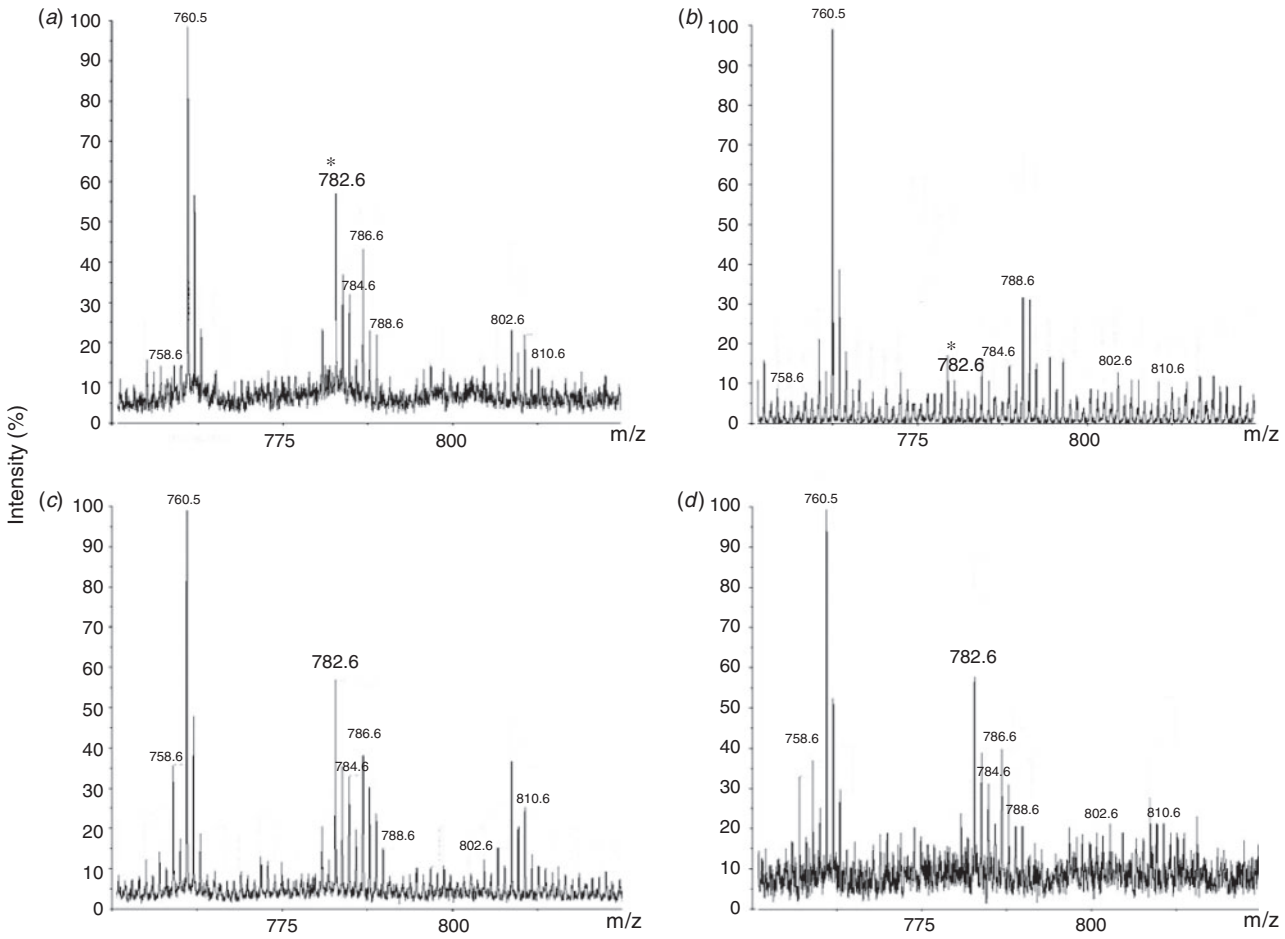

Fig. 6. MALDI mass spectra (positive-ion mode) representative of the lipid profiles of morula-stage bovine embryos produced *in vitro*. (*a*) Holstein, in culture medium containing no added L-carnitine, (*b*) Holstein, in culture medium containing added L-carnitine, (*c*) Jersey, in culture medium containing no added L-carnitine, (*d*) Jersey, in culture medium containing added L-carnitine. *Significant quantitative difference ($P < 0.05$) between Holstein embryo control and L-carnitine treatment.

breeds (Van Soom *et al.* 2003; Leroy *et al.* 2005; Sudano *et al.* 2012). In our study, adding L-carnitine to the culture medium reduced the number of lipid droplets in both Holstein and Jersey embryos. This observation is in agreement with the findings of previous studies (Somfai *et al.* 2011; Takahashi *et al.* 2013; Ghanem *et al.* 2014). This reduction could be due to increased expression of the *ADIPOR2* gene, at least in the Holstein breed. The *ADIPOR2* gene has been described as a major physiological receptor for adiponectin (ADIPOQ; Yamauchi *et al.* 2003; Fischer *et al.* 2010). ADIPOQ is an adipocyte-derived hormone that plays an important role in the stimulation of fatty-acid oxidation and decreases lipid-droplet accumulation as a result of higher mitochondrial activity (Yamauchi *et al.* 2003; Zhou *et al.* 2008; Liu *et al.* 2012; Chen *et al.* 2013). However, L-carnitine was less effective at lowering the lipid-droplet content in Jersey embryos, which was echoed by its impact on embryo colour. We suggest that differences in breed explain this observation. Indeed, we have recently documented a breed effect in lipid content and composition between Holstein and Jersey cows (Baldoceda *et al.* 2016). Previous publications also reported an impact of the genetic background on embryonic lipid content for other breeds, which was found to be higher in the Simmental (*Bos taurus*) and lower in the Nellore breed (*Bos indicus*), *in vivo* as well as *in vitro* (Sudano *et al.* 2012). Our results suggest that the abundance of lipid droplets could explain the lower tolerance of Jersey embryos to cryopreservation compared with Holstein embryos (Steel and Hasler 2004).

A close relationship between mitochondrial activity and lipid-droplet content has been reported previously (Kruip *et al.* 1983; Hyttel *et al.* 1986; Dorland *et al.* 1994; Sturmey *et al.* 2006). The darker cytoplasm observed in bovine embryos obtained *in vitro* thus appears to be related to impaired mitochondrial function

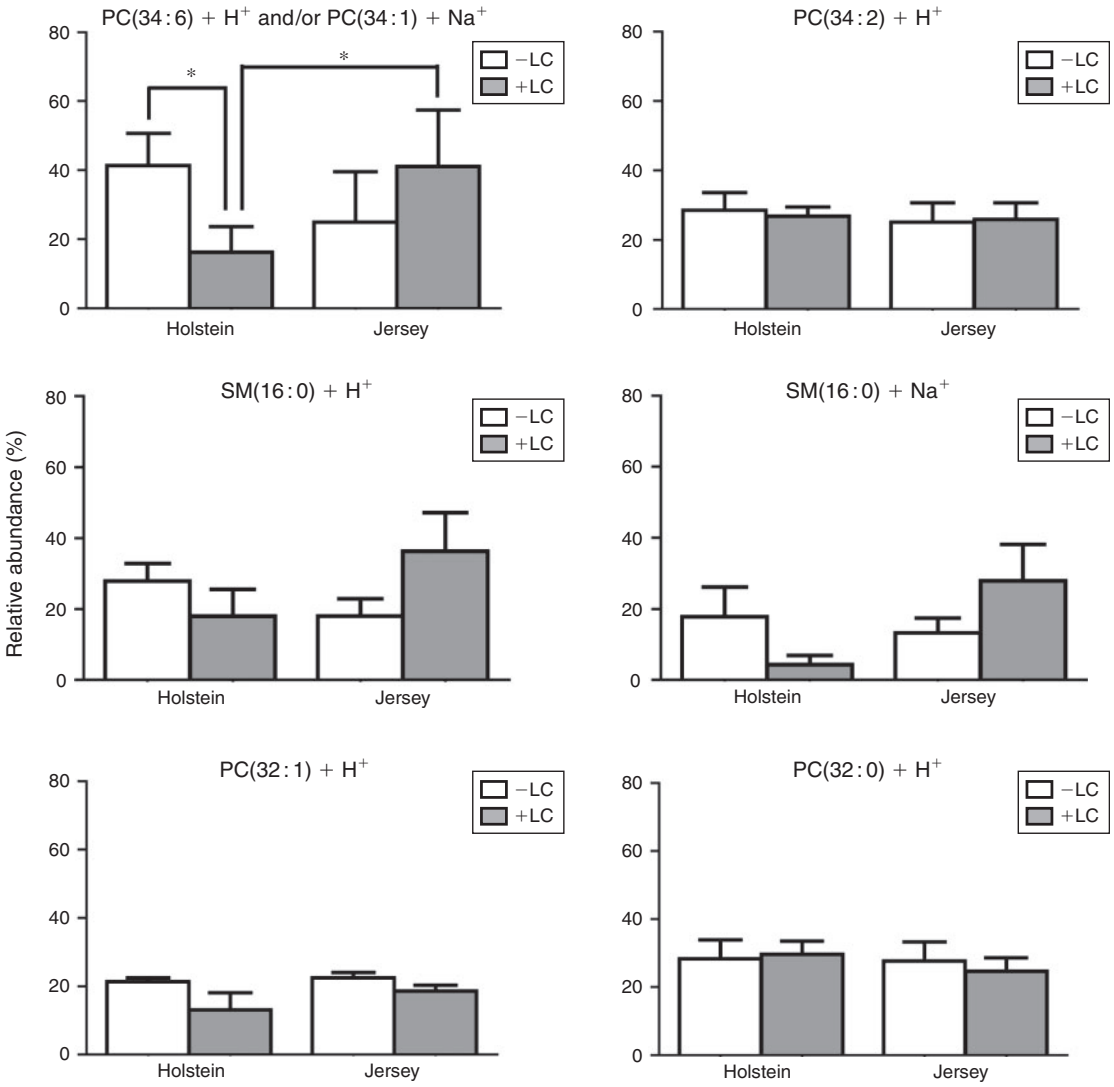

Fig. 7. Relative abundance of lipid species present in morula-stage bovine embryos produced *in vitro*, based on selected ions detected by MALDI mass spectroscopy. PC, phosphatidylcholine; SM, sphingomyelin; −LC, L-carnitine not added to the culture medium; +LC, L-carnitine added to the culture medium. Bars represent mean ± s.e.m. *$P < 0.05$, $n = 12$ embryos per group.

(Thompson *et al.* 1995; Fair *et al.* 2001; Abe *et al.* 2002). Based on our observations of Mitotracker dye intensity, we confirmed that L-carnitine enhanced mitochondrial lipid metabolism, as demonstrated by the reduction in lipid-droplet content and the pale cytoplasm of blastomeres of embryos of either breed. In animal cells, L-carnitine plays an essential role in β-oxidation of long-chain fatty acids by catalysing their transport into the mitochondrial matrix (Kerner and Hoppel 2000). The improvement obtained in embryo mitochondrial activity by adding L-carnitine to the culture medium may thus result from increased β-oxidation. Beta-oxidation generates much of the ATP necessary for embryo development (Ferguson and Leese 1999). Furthermore, expression of the genes *ATP5D* and *CPT2* tended to be stronger in L-carnitine-treated (+LC) Holstein embryos, which confirms that mitochondrial activity was improved. Several studies have related ATP5D (Hong and Pedersen 2003) and CPT2 (Hong and Pedersen 2003; Yao *et al.* 2008) to mitochondrial ATP production during oxidative phosphorylation in eukaryotic cells. However, we observed that L-carnitine supplementation also had a marked effect on mitochondrial activity. The embryo colour and reduced lipid levels observed in this study can therefore be explained in terms of the smaller enhancing effect of L-carnitine on mitochondrial lipid metabolism in the Jersey breed.

Consistent with our present findings, gene expression did not reveal many differences between L-carnitine-treated (+LC) and control (–LC) embryos of the Jersey breed (data not shown). However, L-carnitine did influence the expression of genes associated with carbohydrate and lipid metabolism in Holstein embryos. Based on these results, L-carnitine appears to have a major impact on metabolism in Holstein embryos and only a minor impact in Jersey embryos. Furthermore, expression of the gene *FADS2* tended to be higher in treated (+LC) embryos. It has been demonstrated that FADS2 catalyses the first and rate-limiting step of the biosynthesis and conversion of polyunsaturated fatty acids, which are essential bioactive components of membrane phospholipids (Stoffel *et al.* 2008; Park *et al.* 2009; Stroud *et al.* 2009). These findings are consistent with other studies that have shown that FADS2 appears to play an important role in the modulation of metabolism of saturated as well as unsaturated long-chain CoA acyl esters (Hunt *et al.* 2006; Stoffel *et al.* 2008). FADS2 thus appears to play an important role in modifying membrane fluidity by changing both lipid content and fatty-acid composition. This could explain the different sensitivities to cryopreservation observed in embryos cultured in the presence of L-carnitine (Phongnimitr *et al.* 2013; Takahashi *et al.* 2013).

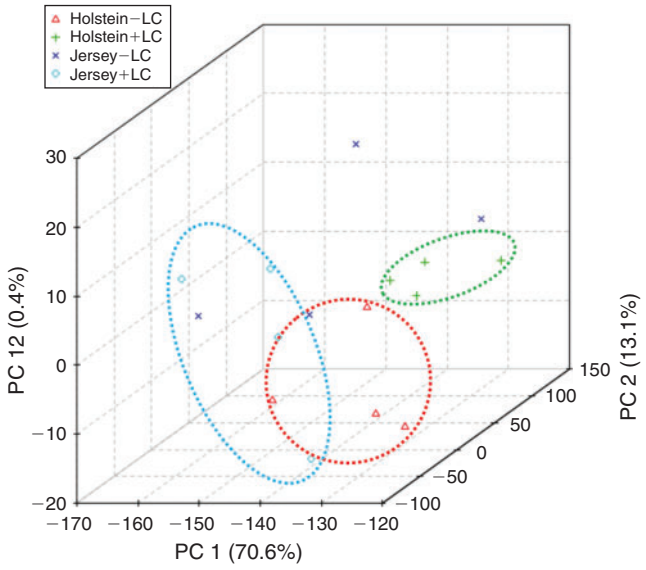

Fig. 8. Three-dimensional representation of principal component analysis (PCA) of the lipid composition of morula-stage bovine embryos produced *in vitro*, based on MALDI-MS data. −LC, L-carnitine not added to the culture medium; +LC, L-carnitine added to the culture medium. $n = 12$ embryos per group.

Lipids play an important role in determining the composition and hence physical properties of cell membranes, which in turn appear to affect the success of cryopreservation (Sata *et al.* 1999; Kim *et al.* 2001; Ferreira *et al.* 2010; Sudano *et al.* 2012). It is also known that environmental conditions influence the lipid profiles of cultured bovine embryos (Sata *et al.* 1999; Kim *et al.* 2001; Ferreira *et al.* 2010). These observations support our findings that the lipid profiles of L-carnitine-treated (+LC) and control (–LC) groups of Holstein embryos did not overlap. However, those of Jersey embryos did overlap, suggesting significant biochemical differences between these two breeds. The unique biochemical characteristics of the Jersey breed in relation to milk fat composition have been described in previous studies (Beaulieu and Palmquist 1995).

The greater abundance of sphingomyelins (lipid ions 16 : 0 $+H^+$ and 16 : 0 $+Na^+$) in Holstein embryos obtained in standard culture medium (–LC) and in Jersey embryos obtained in the modified medium confirmed the differential effects of L-carnitine. Sudano *et al.* (2012) also reported greater abundance of sphingomyelins in association with high lipid content in Simmental embryos obtained *in vitro*. The relevance of these compounds to cattle embryo tolerance of cryopreservation is not known. Phosphatidylcholines containing palmitic (16 : 0), oleic (18 : 1) and linoleic (18 : 2) fatty acids (32 : 0 $+H^+$, 32 : 1 $+H^+$ and 34 : 2 $+H^+$, respectively) have been noted previously among the lipids found in bovine embryos (Sudano *et al.* 2012). It has been suggested that the proportions of these fatty acids play an important role in determining membrane fluidity, which could have a major impact on the success of the cryopreservation process (Pereira *et al.* 2007; Shehab-El-Deen *et al.* 2009; Marei *et al.* 2010; Aardema *et al.* 2011; Van Hoeck *et al.* 2011). We found no notable between-treatment differences in the relative abundances of these phosphatidylcholines in either breed, suggesting that L-carnitine supplementation might not have had much impact on their final levels. However, we noted that treated Holstein embryos contained limited amounts of protonated (34 : 6) or sodiated (34 : 1) phosphatidylcholine. Although their lower abundance was associated with lower lipid content, their role in embryo cryopreservation has not yet been elucidated. However, variations in 34 : 1 phosphatidylcholine have been noted in conjunction with variations in cryopreservation efficiency in the oocytes of different mammalian species (Ferreira *et al.* 2010; Apparicio *et al.* 2012; Sudano *et al.* 2012).

In terms of lipid profile, the responses of Jersey and Holstein embryos produced in culture media enriched with L-carnitine differed considerably, and the reasons for this might be related to breed, based on results presented in this study and others.

In conclusion, the results of the present study show that adding L-carnitine to bovine embryo culture medium can reduce the lipid content of blastomeres, which is anticipated to improve cryopreservation survival. Our results show that L-carnitine supplementation can be used as a treatment to reduce lipid content. From our recent report (Baldoceda *et al.* 2016) and this study, conspicuous differences in phenotype, gene expression and lipid profile were noted between the two dairy breeds and the effect of L-carnitine on embryos of the Jersey breed was overall weaker and more variable. The influence of genetic background on embryonic metabolism and embryo phenotype was thus apparent. Further studies are still needed to improve culture media in order to compensate for the influence of breed on embryonic metabolism.

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References

Aardema, H., Vos, P. L., Lolicato, F., Roelen, B. A., Knijn, H. M., Vaandrager, A. B., Helms, J. B., and Gadella, B. M. (2011). Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol. Reprod.* **85**(1), 62–69. doi:10.1095/BIOLREPROD.110.088815

Abe, H., Otoi, T., Tachikawa, S., Yamashita, S., Satoh, T., and Hoshi, H. (1999). Fine structure of bovine morulae and blastocysts *in vivo* and *in vitro*. *Anat. Embryol. (Berl.)* **199**(6), 519–527. doi:10.1007/S004290050249

Abe, H., Yamashita, S., Satoh, T., and Hoshi, H. (2002). Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol. Reprod. Dev.* **61**(1), 57–66. doi:10.1002/MRD.1131

Apparicio, M., Ferreira, C. R., Tata, A., Santos, V. G., Alves, A. E., Mostachio, G. Q., Pires-Butler, E. A., Motheo, T. F., Padilha, L. C., Pilau, E. J., Gozzo, F. C., Eberlin, M. N., Lo Turco, E. G., Luvoni, G. C., and Vicente, W. R. (2012). Chemical composition of lipids present in cat and dog oocytes by matrix-assisted desorption ionisation mass spectrometry (MALDI- MS). *Reprod. Domest. Anim.* **47**(Suppl 6), 113–117. doi:10.1111/RDA.12003

Baldoceda-Baldeon, L. M., Gilbert, I., Gagne, D., Vigneault, C., Blondin, P., Ferrereia, C., and Robert, C. (2016). Breed specific factors influence embryonic lipid composition: comparison between Jersey and Holstein. *Reprod. Fertil. Dev.* **28**(8), 1185–1196. doi:10.1071/RD14211

Beaulieu, A. D., and Palmquist, D. L. (1995). Differential effects of high-fat diets on fatty-acid composition in milk of Jersey and Holstein cows. *J. Dairy Sci.* **78**(6), 1336–1344. doi:10.3168/JDS.S0022-0302(95)76755-8

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., and Gutierrez-Adan, A. (2010). Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc. Natl. Acad. Sci. USA* **107**(8), 3394–3399. doi:10.1073/PNAS.0913843107

Bousquet, D., Twagiramungu, H., Morin, N., Brisson, C., Carboneau, G., and Durocher, J. (1999). *In vitro* embryo production in the cow: an effective alternative to the conventional embryo production approach. *Theriogenology* **51**(1), 59–70. doi:10.1016/S0093-691X(98)00231-3

Chen, H., Zhang, L., Li, X., Sun, G., Yuan, X., Lei, L., Liu, J., Yin, L., Deng, Q., Wang, J., Liu, Z., Yang, W., Wang, Z., Zhang, H., and Liu, G. (2013). Adiponectin activates the AMPK signalling pathway to regulate lipid metabolism in bovine hepatocytes. *J. Steroid Biochem. Mol. Biol.* **138**, 445–454. doi:10.1016/J.JSBMB.2013.08.013

Diez, C., Heyman, Y., Le Bourhis, D., Guyader-Joly, C., Degrouard, J., and Renard, J. P. (2001). Delipidating *in vitro*-produced bovine zygotes: effect on further development and consequences for freezability. *Theriogenology* **55**(4), 923–936. doi:10.1016/S0093-691X(01)00454-X

Dorland, M., Gardner, D. K., and Trounson, A. O. (1994). Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. *J. Reprod. Fertil. Abstr. Ser.* **13**, 70.

Fair, T., Lonergan, P., Dinnyes, A., Cottell, D. C., Hyttel, P., Ward, F. A., and Boland, M. P. (2001). Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production. *Mol. Reprod. Dev.* **58**(2), 186–195. doi:10.1002/1098-2795(200102)58:2<186::AID-MRD8>3.0.CO;2-N

Ferguson, E. M., and Leese, H. J. (1999). Triglyceride content of bovine oocytes and early embryos. *J. Reprod. Fertil.* **116**(2), 373–378. doi:10.1530/JRF.0.1160373

Ferreira, C. R., Saraiva, S. A., Catharino, R. R., Garcia, J. S., Gozzo, F. C., Sanvido, G. B., Santos, L. F., Lo Turco, E. G., Pontes, J. H., Basso, A. C., Bertolla, R. P., Sartori, R., Guardieiro, M. M., Perecin, F., Meirelles, F. V., Sangalli, J. R., and Eberlin, M. N. (2010). Single embryo and oocyte lipid fingerprinting by mass spectrometry. *J. Lipid Res.* **51**(5), 1218–1227. doi:10.1194/JLR.D001768

Ferreira, C. R., Eberlin, L. S., Hallett, J. E., and Cooks, R. G. (2012). Single oocyte and single embryo lipid analysis by desorption electrospray ionisation mass spectrometry. *J. Mass Spectrom.* **47**(1), 29–33. doi:10.1002/JMS.2022

Fischer, S., Santos, A. N., Thieme, R., Ramin, N., and Fischer, B. (2010). Adiponectin stimulates glucose uptake in rabbit blastocysts. *Biol. Reprod.* **83**, 859–865. doi:10.1095/BIOLREPROD.110.084665

Ghanem, N., Ha, A. N., Fakruzzaman, M., Bang, J. I., Lee, S. C., and Kong, I. K. (2014). Differential expression of selected candidate genes in bovine embryos produced *in vitro* and cultured with chemicals modulating lipid metabolism. *Theriogenology* **82**(2), 238–250. doi:10.1016/J.THERIOGENOLOGY.2014.03.024

González-Serrano, A. F., Pirro, V., Ferreira, C. R., Oliveri, P., Eberlin, L. S., Heinzmann, J., Lucas-Hahn, A., Niemann, H., and Cooks, R. G. (2013). Desorption electrospray ionisation mass spectrometry reveals lipid metabolism of individual oocytes and embryos. *PLoS ONE* **8**(9), e74981. doi:10.1371/JOURNAL.PONE.0074981

Guignot, F. (2005). Cryoconservation des embryons des espèces domestiques. *INRA Prod. Anim.* **18**, 27–35.

Hasler, J. F. (2001). Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. *Theriogenology* **56**(9), 1401–1415. doi:10.1016/S0093-691X(01)00643-4

Hasler, J. F. (2006). The Holstein cow in embryo transfer today as compared to 20 years ago. *Theriogenology* **65**(1), 4–16. doi:10.1016/J.THERIOGENOLOGY.2005.10.002

Hong, S., and Pedersen, P. L. (2003). ATP synthases: insights into their motor functions from sequence and structural analyses. *J. Bioenerg. Biomembr.* **35**(2), 95–120. doi:10.1023/A:1023786618422

Hunt, M. C., Rautanen, A., Westin, M. A., Svensson, L. T., and Alexson, S. E. (2006). Analysis of the mouse and human acyl-CoA thioesterase (ACOT) gene clusters shows that convergent, functional evolution results in a reduced number of human peroxisomal ACOTs. *FASEB J.* **20**(11), 1855–1864. doi:10.1096/FJ.06-6042COM

Hyttel, P., Callesen, H., and Greve, T. (1986). Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. *J. Reprod. Fertil.* **76**(2), 645–656. doi:10.1530/JRF.0.0760645

Kerner, J., and Hoppel, C. (2000). Fatty-acid import into mitochondria. *Biochim. Biophys. Acta* **1486**(1), 1–17. doi:10.1016/S1388-1981(00)00044-5

Kim, J. Y., Kinoshita, M., Ohnishi, M., and Fukui, Y. (2001). Lipid and fatty-acid analysis of fresh and frozen-thawed immature and *in vitro*-matured bovine oocytes. *Reproduction* **122**(1), 131–138. doi:10.1530/REP.0.1220131

Kruip, T. A. M., Cran, D. G., van Beneden, T. H., and Dieleman, S. J. (1983). Structural changes in bovine oocytes during final maturation *in vivo*. *Gamete Res.* **8**, 29–47. doi:10.1002/MRD.1120080105

Leroy, J. L., Opsomer, G., De Vliegher, S., Vanholder, T., Goossens, L., Geldhof, A., Bols, P. E., de Kruif, A., and Van Soom, A. (2005). Comparison of embryo quality in high-yielding dairy cows, in dairy heifers and in beef cows. *Theriogenology* **64**(9), 2022–2036. doi:10.1016/J.THERIOGENOLOGY.2005.05.003

Liu, Q., Yuan, B., Lo, K. A., Patterson, H. C., Sun, Y., and Lodish, H. F. (2012). Adiponectin regulates expression of hepatic genes critical for glucose and lipid metabolism. *Proc. Natl. Acad. Sci. USA* **109**(36), 14 568–14 573. doi:10.1073/PNAS.1211611109

Marei, W. F., Wathes, D. C., and Fouladi-Nashta, A. A. (2010). Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* **139**(6), 979–988. doi:10.1530/REP-09-0503

Murakami, M., Otoi, T., Sumantri, C., and Suzuki, T. (1998). Effects of centrifugation and lipid removal on the cryopreservation of *in vitro*-produced bovine embryos at the eight-cell stage. *Cryobiology* **36**(3), 206–212. doi:10.1006/CRYO.1998.2077

Park, W. J., Kothapalli, K. S., Lawrence, P., Tyburczy, C., and Brenna, J. T. (2009). An alternate pathway to long-chain polyunsaturates: the FADS2 gene product Delta8-desaturates 20:2n-6 and 20:3n-3. *J. Lipid Res.* **50**(6), 1195–1202. doi:10.1194/JLR.M800630-JLR200

Pereira, R. M., Baptista, M. C., Vasques, M. I., Horta, A. E., Portugal, P. V., Bessa, R. J., Silva, J. C., Pereira, M. S., and Marques, C. C. (2007). Cryosurvival of bovine blastocysts is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t,12c CLA). *Anim. Reprod. Sci.* **98**(3–4), 293–301. doi:10.1016/J.ANIREPROSCI.2006.03.015

Phongnimitr, T., Liang, Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetampinich, C., and Parnpai, R. (2013). Effect of L-carnitine on maturation, cryotolerance and embryo developmental competence of bovine oocytes. *Anim. Sci. J.* **84**(11), 719–725. doi:10.1111/ASJ.12067

Plourde, D., Vigneault, C., Lemay, A., Breton, L., Gagne, D., Laflamme, I., Blondin, P., and Robert, C. (2012). Contribution of oocyte source and culture conditions to phenotypic and transcriptomic variation in commercially produced bovine blastocysts. *Theriogenology* **78**(1), 116–131e3. doi:10.1016/J.THERIOGENOLOGY.2012.01.027

Poot, M., Zhang, Y. Z., Kramer, J. A., Wells, K. S., Jones, L. J., Hanzel, D. K., Lugade, A. G., Singer, V. L., and Haugland, R. P. (1996). Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J. Histochem. Cytochem.* **44**(12), 1363–1372. doi:10.1177/44.12.8985128

Rizos, D., Gutierrez-Adan, A., Perez-Garnelo, S., De La Fuente, J., Boland, M. P., and Lonergan, P. (2003). Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance and messenger RNA expression. *Biol. Reprod.* **68**(1), 236–243. doi:10.1095/BIOLREPROD.102.007799

Robert, C., Nieminen, J., Dufort, I., Gagne, D., Grant, J. R., Cagnone, G., Plourde, D., Nivet, A. L., Fournier, E., Paquet, E., Blazejczyk, M., Rigault, P., Juge, N., and Sirard, M. A. (2011). Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Mol. Reprod. Dev.* **78**(9), 651–664. doi:10.1002/MRD.21364

Sata, R., Tsuji, H., Abe, H., Yamashita, S., and Hoshi, H. (1999). Fatty-acid composition of bovine embryos cultured in serum-free and serum-containing medium during early embryonic development. *J. Reprod. Dev.* **45**, 97–103. doi:10.1262/JRD.45.97

Schmittgen, T. D., and Livak, K. J. (2008). Analysing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**(6), 1101–1108. doi:10.1038/NPROT.2008.73

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**(7), 671–675. doi:10.1038/NMETH.2089

Seidel, G. E., Jr. (2006). Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* **65**(1), 228–235. doi:10.1016/J.THERIOGENOLOGY.2005.09.025

Shehab-El-Deen, M. A., Leroy, J. L., Maes, D., and Van Soom, A. (2009). Cryotolerance of bovine blastocysts is affected by oocyte maturation in media containing palmitic or stearic acid. *Reprod. Domest. Anim.* **44**(1), 140–142. doi:10.1111/J.1439-0531.2008.01084.X

Somfai, T., Kaneda, M., Akagi, S., Watanabe, S., Haraguchi, S., Mizutani, E., Dang-Nguyen, T. Q., Geshi, M., Kikuchi, K., and Nagai, T. (2011). Enhancement of lipid metabolism with L-carnitine during *in vitro* maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod. Fertil. Dev.* **23**(7), 912–920. doi:10.1071/RD10339

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, Article3. doi:10.2202/1544-6115.1027

Steel, R., and Hasler, J. F. (2004). 120 Pregnancy rates resulting from transfer of fresh and frozen Holstein and Jersey embryos. *Reprod. Fertil. Dev.* **16**, 182–183. [Abstract]. doi:10.1071/RDV16N1AB120

Stoffel, W., Holz, B., Jenke, B., Binczek, E., Gunter, R. H., Kiss, C., Karakesisoglou, I., Thevis, M., Weber, A. A., Arnhold, S., and Addicks, K. (2008). Delta6-desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J.* **27**(17), 2281–2292. doi:10.1038/EMBOJ.2008.156

Stroud, C. K., Nara, T. Y., Roqueta-Rivera, M., Radlowski, E. C., Lawrence, P., Zhang, Y., Cho, B. H., Segre, M., Hess, R. A., Brenna, J. T., Haschek, W. M., and Nakamura, M. T. (2009). Disruption of *FADS2* gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* **50**(9), 1870–1880. doi:10.1194/JLR.M900039-JLR200

Sturmey, R. G., O'Toole, P. J., and Leese, H. J. (2006). Fluorescence resonance energy transfer analysis of mitochondrial : lipid association in the porcine oocyte. *Reproduction* **132**(6), 829–837. doi:10.1530/REP-06-0073

Sudano, M. J., Santos, V. G., Tata, A., Ferreira, C. R., Paschoal, D. M., Machado, R., Buratini, J., Eberlin, M. N., and Landim-Alvarenga, F. D. (2012). Phosphatidylcholine and sphingomyelin profiles vary in *Bos taurus indicus* and *Bos taurus taurus in vitro*- and *in vivo*-produced blastocysts. *Biol. Reprod.* **87**(6), 130. doi:10.1095/BIOLREPROD.112.102897

Takahashi, T., Inaba, Y., Somfai, T., Kaneda, M., Geshi, M., Nagai, T., and Manabe, N. (2013). Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced *in vitro*. *Reprod. Fertil. Dev.* **25**(4), 589–599. doi:10.1071/RD11262

Tata, A., Sudano, M. J., Santos, V. G., Landim-Alvarenga, F. D., Ferreira, C. R., and Eberlin, M. N. (2013). Optimal single-embryo mass spectrometry fingerprinting. *J. Mass Spectrom.* **48**(7), 844–849. doi:10.1002/JMS.3231

Thompson, J. G., Gardner, D. K., Pugh, P. A., McMillan, W. H., and Tervit, H. R. (1995). Lamb birth weight is affected by culture system utilised during *in vitro* pre-elongation development of ovine embryos. *Biol. Reprod.* **53**(6), 1385–1391. doi:10.1095/BIOLREPROD53.6.1385

Van Hoeck, V., Sturmey, R. G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H. J., Bols, P. E., and Leroy, J. L. (2011). Elevated non-esterified fatty-acid concentrations during bovine oocyte maturation

compromise early embryo physiology. *PLoS ONE* **6**(8), e23183. doi:10.1371/JOURNAL.PONE.0023183

Van Soom, A., Mateusen, B., Leroy, J., and De Kruif, A. (2003). Assessment of mammalian embryo quality: what can we learn from embryo morphology? *Reprod. Biomed. Online* **7**(6), 664–670. doi:10.1016/S1472-6483(10)62089-5

Xia, J., Psychogios, N., Young, N., and Wishart, D. S. (2009). MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* **37**(Suppl 2), W652–W660. doi:10.1093/NAR/GKP356

Yamashita, S., Abe, H., Itoh, T., Satoh, T., and Hoshi, H. (1999). A serum-free culture system for efficient *in vitro* production of bovine blastocysts with improved viability after freezing and thawing. *Cytotechnology* **31**(1–2), 123–131. doi:10.1023/A:1008044827145

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N. H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., and Kadowaki, T. (2003). Cloning of adiponectin receptors that mediate anti-diabetic metabolic effects. *Nature* **423**(6941), 762–769. doi:10.1038/NATURE01705

Yao, D., Mizuguchi, H., Yamaguchi, M., Yamada, H., Chida, J., Shikata, K., and Kido, H. (2008). Thermal instability of compound variants of carnitine palmitoyltransferase II and impaired mitochondrial fuel utilisation in influenza-associated encephalopathy. *Hum. Mutat.* **29**(5), 718–727. doi:10.1002/HUMU.20717

Zhou, M., Xu, A., Tam, P. K., Lam, K. S., Chan, L., Hoo, R. L., Liu, J., Chow, K. H., and Wang, Y. (2008). Mitochondrial dysfunction contributes to the increased vulnerabilities of adiponectin-knockout mice to liver injury. *Hepatology* **48**(4), 1087–1096. doi:10.1002/HEP.22444