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 to *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 breed 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 accounted for 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.
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 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 focused 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 three 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 post-oestrus. Administration of follicle-stimulating hormone (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) was begun 36 h after follicular removal. Follicle-stimulating hormone was injected twice a day in doses of 40 mg for a total of 240 mg (six injections). Forty-eight hours after the final injection, the ultrasound-based pick-up procedure was performed using an 18G 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 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 % CO₂, 20 % O₂ atmosphere. The maturation medium contained TCM199 (Gibco 11150-059, Invitrogen, Burlington, ON, Canada), 10 % foetal calf serum (Sterile Foetal Bovine Serum for Cell Culture, Medicorp, Montreal, QC, Canada), 200 µM pyruvate, 50 µg/mL gentamycin and 0.1 µg/mL follicle-stimulating hormone (Gonal-f, Serono Canada Inc., Mississauga, ON, Canada).

In vitro fertilization

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 (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 fertilization 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 x 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 fertilize 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 (Sigma-Aldrich, #C0158) (the “treated” or “ + LC ” group) or without (the “control” or “ – LC” group). The SOF media was a standard culture medium containing amino acids and 0.4 % fatty-acid-free bovine serum albumin (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 (#8410, Sigma). The culture dishes were incubated at 38.5 °C in a humidified atmosphere containing 6.5 % CO₂, 5 % O₂ and 88.5 % N₂. Ten embryos were transferred to each fresh droplets (10 µL) at 72 h and 120 h (20 µL) after fertilization 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 categorized according to IETS system for comparison purposes. Samples were produced using seven independent in vitro production runs. The COCs from different donor cows were pooled within respective 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 = eight 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 minutes at 38 °C in 5 % CO₂. 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 to other dyes, due to high co-localization 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 minutes in SOF containing 3 μg/mL of the lipid-specific dye 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (Bodipy 493/503, Molecular Probes, Eugene, OR, USA) in order to stain cytoplasmic lipid droplets. To label nuclei, embryos were incubated for 10 min at room temperature in SOF containing 1 μg/mL of Hoechst blue dye 33342. Non-fixed specimens were washed three times in SOF and mounted on the microscope slide in the same media using 120 μm thick spacers (Sigma-Aldrich, #GBL654008).

Confocal microscopy

Bright field, confocal and epifluorescence images were acquired using a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada) with a 60x/1.20 water-
immersion objective. Bright field images of morula embryo morphological phenotype were recorded in grey scale photos 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 x 512 pixels. The respective excitation and emission wavelengths were as follows: Bodipy (488 nm, 515-530 nm), and the Mitotracker Red (555nm, 605-675nm). All the settings were the same for all samples. Mitochondrial activity was the recorded as an epifluorescence image of CMX-rosamine dye in grey scale and carried out using Nikon TE2000 microscope quipped with epifluorescence illumination and appropriate filters, which were G-2a for the Mitotracker Red. Digital images of stained embryos were viewed with the Plan-Apochromat 40x lens (NA = 1.2) at 10 %utilizable laser intensity (maximum power: 1.2 W, output: 25 % of the maximum tube current) and a HFT dichroic beam splitter (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 x 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 (Abramoff et al. 2004). Results are expressed in arbitrary units (AU) as the mean fluorescence intensity of all samples within a group. Measurements of the 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 5 pixels (which represents 0.5 μm²) 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) 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. RNA was eluted in 11 μl of elution buffer and 1 μl was used to measured the quality and concentration of extracted RNA using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA PicoLab Chip (Agilent Technologies). Only RNA of very good quality (RIN over 8) was used for the amplification.

Mitochondrial DNA quantification and quantitative RT-PCR validation were performed using individual blastocysts (n = four per group). Simultaneous extraction of total genomic DNA and RNA were done using the AllPrep DNA/RNA Micro Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer’s instructions. The DNA
and RNA of each blastocyst were eluted in 30 µl and 10 µl volume respectively. The DNA extract was used for mitochondrial DNA quantification and total RNA was reverse transcribed and used for quantitative RT-PCR (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) 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 MgCl₂, 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Laval, QC, Canada) 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 sec, 5 sec (12S rRNA) at 58 °C or 60 °C (Mx1), followed by 72 °C for 20 sec and 76 °C (12S rRNA) or 85 °C (Mx1) for 5 sec. The presence of amplicons was verified using melting curve analysis. 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 bioanalyzer 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 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 hybridized 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. A simple direct comparison between control (-LC) and treated (+LC) embryos from each breed was performed. Microarrays slides were then washed and scanned with the PowerScanner (Tecan, Männedorf, Switzerland) and analyzed with Array-Pro Analyzer 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) under accession number GSE62595. Briefly, data intensity files were analyzed by FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray), where raw data corrected by background subtraction were preprocessed using Lowess intra-array normalization and Quantile inter-array normalization. Statistically significant variations were detected using Limma (Bioconductor). Differences in gene expression were considered 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 obtain 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 (ADIPOR2: adiponectin receptor 2, ATP5D: ATP synthase H+ transporting mitochondrial FI complex delta subunit, CPT2: carnitine palmitoyltransferase 2, ACOT4: acyl-CoA thioesterase 4 and FADS2: fatty acid desaturase 2) were designed using the Primer3 Web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthetized 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, Laval, QC, Canada). Real-time measurements were performed in a LightCycler 2.0 apparatus (Roche Diagnostics). GeneBank 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 (CT) method was then used to quantify expression levels (Schmittgen and Livak 2008). Quantification was normalized relative to the level of beta-actin expression (endogenous control). The CT of beta-actin was subtracted from the CT of the gene to obtain ΔCT. For the calculation of ΔΔCT, the highest sample ΔCT value (i.e. from the sample with the lowest target expression) was subtracted from all other
ΔCT values. The change in the relative level of gene expression of the target was calculated as $2^{-\Delta\Delta CT}$.

Lipid profile analyses by MALDI-MS

The lipid profile of intact embryos was analysed using a mass spectroscopy (matrix-assisted laser desorption/ionization or MALDI) procedure described previously (Gonzalez-Serrano et al. 2013) with some modifications. Briefly, each biological replicate was composed of three morula-stage embryos. Analysis was done on 4 or 5 biological replicates per group. Embryos were first washed three times in 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 (HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA) in ultrapure water (Millipore, Billerica, MA, USA) and washed three times in 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 on 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, Ontario, Canada) equipped with a 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 60-90s laser shots, until the signal from that location disappeared due to ablation of the
sample. MALDI-MS data based 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 m/z values corresponding to lipids. After attribution, only the m/z values that were clearly above background levels were included in the principal component analysis, which was performed using Pirouette v.3.11 (Infometrix Inc., Woodinville, WA, USA). Ion fragmentation patterns obtained in previous studies (Ferreira et al. 2010; Ferreira et al. 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 v.3.11 (Infometrix, Inc.) and the MetaboAnalyst website (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 showed in Figure 1. The cytoplasm of blastomeres in the treated group (+LC) appears pale compared the control (-LC) group regardless of the breed. Some embryos respond more profoundly to the treatment than others as some embryos become very pale. The underlying mechanism for this variability to react 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 Figure 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 association with the treatment even when the breeds were considered together (p < 0.05, Figure 3A), while the average volume tended to be lower (Figure 3B) 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, Figure
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 (Figure 4B).

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 genes 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 signaling 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 the L-carnitine treatment (Figure 5).

Effect of L-carnitine on the lipid profiles of Holstein and Jersey embryos obtained in vitro
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can provide directly a lipid fingerprint of a single intact cattle embryo, in particular the profile of phospholipids such as phosphatidylcholines and sphingomyelins (Ferreira et al. 2010; Apparicio et al. 2012; Ferreira 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). The representative lipid profile of each group is shown in Figure 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 the control (-LC) embryos of the Holstein breed (Figure 7). This phosphatidylcholine was also significantly less abundant in Holstein than in Jersey embryos subjected to the L-carnitine treatment (Figure 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 (Figure 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) among the four groups (Figure 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 respectively with the Holstein control group and with all treatments (Figure 8).
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 the 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 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 (Bladoceda et al., in press). Previous publications also reported an impact of the genetic background on embryonic lipid content for other breeds, which was found higher in Simmental (*Bos taurus*) and lower in Nellore (*Bos indicus*), *in vivo* as well as *in vitro* (Sudano *et al.* 2012). Our results suggest that abundance of lipid droplets could explain the lower tolerance of Jersey embryos to cryopreservation compared to 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 related to impaired mitochondrial function (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 beta-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 confirm that mitochondrial activity was improved. Several studies have related ATP5D (Hong and Pendersen, 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 has a marked effect on mitochondrial activity. The embryo colour and reduced lipid levels observed in this study therefore can 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 appear 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).

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.
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 (respectively 32:0 + H⁺, 32:1 + H⁺ and 34:2 + H⁺) 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 the bovine embryo culture medium can reduce lipid content of blastomere which is anticipated as a mean to improve survival to cryopreservation. Our results show that L-carnitine supplementation can be used as a treatment to reduce lipid content. From our recent report (Baldoceda et al., in press) 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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Hasler, J.F. (2006) The Holstein cow in embryo transfer today as compared to 20 years ago. Theriogenology 65(1), 4-16


Figure 1. Phase contrast images of morula-stage bovine embryos produced *in vitro*, Holstein (Ho), no L-carnitine (-LC) added, culture medium enriched with L-carnitine (+LC), Jersey (Je), no L-carnitine added (-LC), culture medium enriched with L-carnitine (+LC).
Figure 2. 3D orthogonal projection of confocal images of active mitochondria labeled with Mitotracker Red (red), lipid droplets labeled with Bodipy 493/503 (green) and DNA labeled 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).
**Figure 3.** Lipid droplets (LD) in morula-stage bovine embryos produced *in vitro* A) Number, B) Mean volume in femtolitres, -LC = L-carnitine not added to culture medium, +LC = L-carnitine added to culture medium. Bars represent mean ± SEM. *Significantly different (P < 0.05).
Figure 4. Fluorescence intensity (in arbitrary units, AU) of Mitotracker Red (A) and ratio of mitochondrial (mt) to nuclear (n) DNA (B) in bovine embryos produced in culture media containing no added L-carnitine (-LC) and containing added L-carnitine (+LC). Medium containing carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a negative control. Bars represent mean ± SEM. *Significantly different (P < 0.05).
Figure 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 = L-carnitine not added to the culture medium, +LC = L-carnitine added to the culture medium. Bars represent mean ± SEM. Quantities are normalized relative to endogenous beta-actine transcripts.
Figure 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 not containing added L-carnitine, B) Holstein, in culture medium containing added L-carnitine, C) Jersey, in culture medium not containing added L-carnitine, D) Jersey, in culture medium containing added L-carnitine. *Significant quantitative difference ($P < 0.05$) between Holstein embryos control and L-carnitine treatment.
Figure 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 ± SEM.

*Significantly different (P < 0.05, n = 12 embryos per group).
Figure 8. Three-dimensional representation of principal component (PC) analysis 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).
**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

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Accession</th>
<th>Primer sequences</th>
<th>Annealing (°C)</th>
<th>Acquisition (°C)</th>
<th>Product size (bp)</th>
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<tr>
<td>ADIPOR2</td>
<td>NM_001040499</td>
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<td>ATP5D</td>
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<td>12s</td>
<td>J0_1394</td>
<td>TCGATAAACCACGATAACC TTCGTGCTTGATTCTCTTG</td>
<td>58</td>
<td>76</td>
<td>186</td>
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Table 2. The most significant phosphatidylcholine (PC) and sphingomyelin (SM) ions identified based on MALDI-MS data obtained from individual bovine embryos

<table>
<thead>
<tr>
<th>m/z</th>
<th>Lipid ion (C atoms: unsaturation)</th>
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<tr>
<td>703.5</td>
<td>[SM (16:0) + H]⁺</td>
</tr>
<tr>
<td>725.5</td>
<td>[SM (16:0) + Na]⁺</td>
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<tr>
<td>732.5</td>
<td>[PC (32:1) + H]⁺</td>
</tr>
<tr>
<td>734.5</td>
<td>[PC (32:0) + H]⁺</td>
</tr>
<tr>
<td>758.6</td>
<td>[PC (34:2) + H]⁺</td>
</tr>
<tr>
<td>760.5</td>
<td>[PC (34:1) + H]⁺</td>
</tr>
<tr>
<td>782.6</td>
<td>[PC (34:6) + H]⁺, [PC (34:1) + Na]⁺</td>
</tr>
<tr>
<td>784.6</td>
<td>[PC (34:0) + Na]⁺</td>
</tr>
<tr>
<td>786.6</td>
<td>[PC (36:2) + H]⁺</td>
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<tr>
<td>788.6</td>
<td>[PC (36:1) + H]⁺</td>
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<td>802.6</td>
<td>[PC (36:5) + Na]⁺</td>
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<tr>
<td>810.6</td>
<td>[PC (38:4) + H]⁺, [PC (36:1) + Na]⁺</td>
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Identification is based on the collision induced dissociation database and on earlier studies (Ferreira et al., 2010; Sudano et al., 2012).