Contents
The aim of the present study was to compare the lipid profile in oocytes of indicus and 1/2 indicus × taurus cows with high and low antral follicle count (AFC)/oocyte yields. After an OPU procedure (D0), antral follicles ≥3 mm were counted by ultrasonography (D4, 19, 34, 49, 64), and cows were assigned to groups with either high AFC (≥30 follicles; indicus, NH group; 1/2 indicus × taurus, AH group) or low AFC (≤15 antral follicles; indicus, NL group; 1/2 indicus × taurus, AL group). The lipid profiles of the oocytes were determined by MALDI-MS. For GI, GII and GIII oocytes, the indicus samples tend to cluster separately from the 1/2 indicus × taurus samples. The lipid species [PC (P-38:5) + H]+ and/or [PC (P-36:2) + Na]+, [PC (38:2) + H]+, [PC (38:5) + Na]+ and [TAG (60:8) + NH]+ were more abundant in indicus (NH and NL groups) than 1/2 indicus × taurus. The higher lipid content in the indicus oocytes likely reflects differences in the rate of lipid metabolism and may contribute to oocyte competence and embryo development.

Introduction
Bos indicus cows usually produce more oocytes than Bos taurus donors (Machado et al. 2006; Pontes et al. 2009; Pontes et al. 2010). Good oocyte yield can be obtained from B. indicus and B. taurus crosses (Pontes et al. 2010). The adaptability of indicus-taurus cross-bred females to tropical climates has encouraged their use in tropical and subtropical areas. There is, however, great variation in oocyte production among individuals (Pontes et al. 2009, 2011). Although it is possible to produce more embryos and pregnancies following OPU/in vitro embryo production (IVP) than superovulation/embryo transfer (ET) in indicus cows (Pontes et al. 2009), individual variation is observed in the oocyte production of cows with indicus and indicus-taurus backgrounds. There is, however, little information about the oocyte differences between these cattle types.

Triacylglycerides (TAG) predominate the lipid content of the cytoplasm of mammalian cells, forming lipid droplets (Ferguson and Leese 1999; Hulbert 2003; Aardema et al. 2011; McKeegan and Sturmey 2011). These lipids are crucial for oocytes and embryos because they function as a form of energy storage (Sturmey et al. 2011). However, reduced triacylglyceride and cholesterol stores are associated with a lower developmental capacity of oocytes to undergo maturation, fertilization and early cleavage (Kim et al. 2001; Ferguson and Leese 1999). These trends suggest that lipids are used as a cellular energy source. In addition, lipid–organellar interactions likely occur during oocyte maturation because the mitochondria, lipids and smooth endoplasmic reticulum remain in clusters (Fleming and Saacke 1972; Hyttel et al. 1997).

Among the analytical techniques for lipid analysis of mammalian oocytes and pre-implantation embryos, mass spectrometry (MS) is emerging as a powerful tool. MS has been used to obtain lipid structural information from individual or small pools of oocytes (2–5) from human, bovine, sheep, mouse, canine and feline species as well as from individual pre-implantation embryos obtained from cattle and mice (Ferreira et al. 2010, 2012a,b; Apparicio et al. 2012; Sudano et al. 2012). The most commonly used technique for lipid profiling in oocytes and embryos is matrix-assisted desorption/ionization (MALDI; Ferreira et al. 2010). A more direct approach using an ambient desorption/ionization technique (DESI) has also been demonstrated recently (Ferreira et al. 2012b). MALDI-MS has been used under conditions that favour the detection of PC, SM and TAG.

In the present study, MALDI-MS lipid profiles of oocytes from B. indicus and 1/2 indicus × taurus donors with high and low oocyte yields were compared to identify significant lipid structural differences. We predict that lipids have potential as biomarkers for early diagnosis of high-oocyte-yield animals and that lipid analysis may contribute to the elucidation of the molecular mechanisms underlying higher oocyte yields.

Materials and Methods
Animals
Nelore females (B. indicus, n = 20) and 1/2 Nelore × Angus females (indicus/taurus, n = 20) that were 6–8 years old and had a body weight of 450 ± 10 kg and a...
body condition score of 3.5 ± 0.5 (scale, 1–5; Lowman et al. 1976) were subjected to follicular aspiration on random days during the oestrous cycle (D0) to promote the ablation of all follicles ≥3 mm and the beginning of a new follicular wave. Animals were maintained in a Brachiaira brizantha pasture supplemented with mineral salt ad libitum. Some of the animals were selected for lipid profile evaluation based on the antral follicle count (described below).

Antral follicular counting

Ovaries from each animal were monitored with a 7.5-MHz convex intravaginal array transducer (Aquila PRO; Pie medical, Maastricht, The Netherlands) at 15-d intervals after OPU (Days 4, 19, 34, 49, and 64), and antral follicles were counted as described previously (Burns et al. 2005; Ireland et al. 2008). Each ovary was scanned from end to end to identify positions of the CL and antral follicles ≥3 mm, which were drawn on an ovarian map. Each follicle was measured, and the diameter was averaged and recorded next to the appropriate follicle on each ovarian map. The antral follicle count (AFC – total number of antral follicles ≥3 mm in diameter) per pair of ovaries was determined for each animal. After five ultrasound evaluations performed by the same operator, females were assigned to two groups based on the number of antral follicles ≥3 mm in diameter: females with a consistently high (≥30 follicles; indicus, n = 3; 1/2 indicus × taurus, n = 5) or low AFC (≤15 follicles; indicus n = 4; 1/2 indicus × taurus, n = 4) in all ultrasound scans. Animals with intermediate AFC (>15 and ≤45 follicles) were not studied further.

Follicular aspiration and oocyte recovery

Oocytes were obtained by follicular aspiration from indicus and 1/2 indicus × taurus females with high and low AFC. Briefly, each visible follicle was aspirated using a real-time B-mode ultrasound scanner (Aquila PRO; Pie medical), a 7.5-MHz convex array transducer fitted into the intravaginal device (Pie Medical) and a stainless steel guide. Follicular puncture was performed using a disposable 19-gauge 1/2″ hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-ml conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm id). Aspiration was performed by using a disposable 19-gauge 1/2″ hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-ml conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm id). Aspiration was performed by using a vacuum pump (WTA, Watanabe, Cravinhos, São Paulo, Brazil) with a negative pressure of 75 mm Hg. The collection medium was phosphate buffer solution (PBS, Nutricell, Campinas, SP, Brazil) with 10 000 IU/1 sodium heparin (Sigma H-3149; Sigma-Aldrich, St. Louis, MO, USA). Immediately after recovery, the aspirated material was washed and filtered through an Emecon embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with a phosphate buffer solution (PBS, Nutricell). The cumulus oocyte complexes (COCs) were classified according to the presence of cumulus cells and the oocyte quality using the following criteria: (i) COCs from pre-antral follicles, (ii) grade I (GI), (iii) grade II (GII), (iv) grade III (GIII), (v) denuded, (vi) expanded and (vii) atretic oocytes.

Experimental design

Of the 83 oocytes recovered by OPU, only oocytes classified as GI, GII and GIII (total n = 53; 23 mass spectra) were considered in the lipid profile and statistical analysis for the breed and oocyte yield comparison. The four experimental groups were defined as follows: oocytes from indicus cows with high AFC (NH group; n = 31; 10 mass spectra), indicus cows with low AFC (NL group; n = 5, 3 mass spectra), 1/2 indicus × taurus cows with high AFC (AH group; n = 12, 6 mass spectra) and 1/2 indicus × taurus with low AFC (AL group, n = 5, 4 mass spectra).

Lipid analysis by MALDI-MS

Sample preparation

Each indicus or 1/2 indicus × taurus cumulus oocyte complex with cumulus cells and homogeneous cytoplasm was denuded of cells by gentle pipetting in 0.5% hyaluronidase. All oocytes were washed three times in drops of a PBS solution containing 0.1% polystyrene alcohol and stored in microtubes containing 100 μl of a 50% aqueous methanol solution (ACS/HPLC grade; Burdick and Jackson, Muskegon, MI, USA)/ultrapure water (Millipore, Bedford, MA, USA) at ~8°C for transportation and immediate analysis. Sample preparation involved placing each oocyte in a given spot of the target plate under the stereomicroscope. Samples were allowed to dry at room temperature, and their location was recorded to place the laser at the correct location during analysis. Just before analysis, 1 μl of 1.0 mM 2,5-dihydroxybenzoic acid (DHB) in methanol was placed in each target spot and allowed to dry at room temperature.

MALDI-MS data acquisition

Oocytes were washed in ultrapure H2O/methanol 1:1 (v/v) and placed (one to four oocytes of the same quality/spot) in the MALDI target plate. After drying at room temperature, samples were then covered with 1 μl of 2,5-DHB 0.5 μl dissolved in pure methanol as the MALDI matrix. The MALDI target plate was then immediately placed in a Q-ToF Premier (Synapt HDMS) mass spectrometer (Waters, Manchester, UK) equipped with a 200 Hz solid-state laser in the m/z 700–1200 range, operated in the reflection and QTOF modes. The instrument operating conditions were a laser energy of 250 a.u. and a sample plate voltage of 20 V. Unmodified (i.e. no extraction procedure) oocytes were directly analysed. All mass spectra were manually collected for approximately 1 min in positive ion mode. Mass spectra were processed with MassLynx 4.1 software (Waters Corp, Milford, MA, USA).

Statistical analysis

A list of the relative ion abundances of the samples organized in columns was constructed using Excel software. The data were uploaded as a .csv file in the online software MetaboAnalyst (Xia et al. 2009; Xia et al. 2011a; Xia et al. 2012) as a peak intensity table in unpaired columns. Because relative ion abundances...
(and not absolute ion counts) were used, no further normalization was performed. The data were submitted to a partial least squares-discriminant analysis (PLS-DA). This analysis is a supervised clustering method in which previous knowledge of the classes is used during the classification process. PLS-DA projects the data into a low-dimensional space that maximizes the separation between the different groups of data in few dimensions (called latent variables), which are ranked by how well they explain the variance of the data. PLS-DA in MetaboAnalyst produces the variable importance measures by weighting a sum of squares of the PLS loadings that takes into account the amount of explained variance of each component (Xia and Wishart 2011b,c). The average values of lipid ion abundance and the standard deviation were calculated using Excel software.

Results

Bovine oocytes recovered by OPU were classified according to their quality in (i) COCs from pre-antral follicles, (ii) GI, (iii) GII, (iv) GIII and (v) expanded oocytes. When only information on oocyte quality regardless of breed and oocyte yield/antral follicle count – was plotted in PLS-DA analysis, the pre-antral and expanded samples were separated from the other groups (Fig. 1). COCs from the pre-antral follicles presented the lowest abundance of SM \([\text{SM} (16:0) + \text{Na}^+]\) at \(m/z\) 725.4, PC \([\text{PC} (38:2) + \text{H}]^+\) at \(m/z\) 814.8, plasmalogen \([\text{PC} (P-38:5) + \text{H}]^+\) and/or \([\text{PC} (P-36:2) + \text{Na}]^+\) at \(m/z\) 792.8, and \([\text{PC} (38:7) + \text{H}]^+\) and/or \([\text{PC} (36:4) + \text{Na}]^+\) at \(m/z\) 804.4; and higher abundances of \([\text{PC} (32:1) + \text{Na}]^+\) at \(m/z\) 754.4 (Table 1), whereas the expanded oocytes presented higher abundances of \(m/z\) 725.4, 814.8, 792.8 and 804.4 and lower abundances of \(m/z\) 754.4. The lipid profiles of oocytes from GI, GII and GIII were similar and could not be distinguished based on their lipid profiles. Figure 2 shows representative MALDI mass spectra. To eliminate interference due to oocyte quality in further PLS-DA analysis of the four experimental groups, only GI, GII and GIII oocytes were considered. When considering only GI, GII and GIII oocytes, indicus samples tended to cluster separately in the PLS-DA plot from 1/2 indicus × taurus samples, particularly the NH group (Fig. 3), whereas 1/2 indicus × taurus

<table>
<thead>
<tr>
<th>(m/z)</th>
<th>Attribution [class (number of carbons/unsaturated carbons) + proton/adduct] (^a)</th>
</tr>
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<tbody>
<tr>
<td>725.4</td>
<td>([\text{SM} (16:0) + \text{Na}]^+)</td>
</tr>
<tr>
<td>754.4</td>
<td>([\text{PC} (32:1) + \text{Na}]^+)</td>
</tr>
<tr>
<td>792.4</td>
<td>([\text{PC} (P-38:5) + \text{H}]^+), ([\text{PC} (P-36:2) + \text{Na}]^+) (^b)</td>
</tr>
<tr>
<td>804.4</td>
<td>([\text{PC} (38:7) + \text{H}]^+) and/or ([\text{PC} (36:4) + \text{Na}]^+) (^a)</td>
</tr>
<tr>
<td>814.4</td>
<td>([\text{PC} (38:2) + \text{H}]^+) (^b)</td>
</tr>
<tr>
<td>830.8</td>
<td>([\text{PC} (38:5) + \text{Na}]^+) (^b)</td>
</tr>
<tr>
<td>990.8</td>
<td>([\text{TAG} (60:8) + \text{NH}_4]^+) (^b)</td>
</tr>
</tbody>
</table>

SM, sphingomyelin; PC, phosphatidylcholine; TAG, triacylglycerol.

An error of up to 0.3 Da was considered acceptable due to the TOF resolution. The ‘P’ prefix is used for the 1Z-alkenyl ether (Plasmalogen) substituent.

\(^a\) Attribution has been performed based on our previous work using the same methodology of lipid analysis in mammalian oocytes (Ferreira et al. 2010; Sudano et al. 2012).

\(^b\) Attribution has been performed based on LipidMaps (The LIPID MAPS Lipidomics Gateway, http://www.lipidmaps.org/) considering that MALDI in positive ion mode with DHB as an organic matrix is highly selective for SM, PC and TAG detection.
oocytes (AH and AL group) were mixed. The most important ions that explain this variability are those at m/z 792.8, 814.8, [PC (38:5) + Na]⁺, at m/z 830.8, and [TAG (60:8) + NH4]⁺ at m/z 990.8, which were more abundant in the NH and NL groups than in the AH and AL groups.

The relevance of the four most abundant ions present in indicus oocytes was further confirmed by plotting the

Fig. 2. Representative MALDI-MS of (a) expanded oocytes; (b) GI; (c) GII; (d) GIII oocytes; (e) COCs from pre-antral follicles.
respective relative ion abundances in individual samples (Fig. 4). Figure 5 shows representative spectra for the four experimental groups.

Discussion

Herein, we report the first comparative MALDI-MS-based study of lipid profiles of immature oocytes recovered by OPU from B. indicus and 1/2 indicus-taurus females with remarkably high or low antral follicle count/oocyte yield.

Lipids have been studied and may represent reliable biomarkers for oocyte development because they play many significant roles, such as energy storage and cell structure (by tuning the physical properties of the cell; Kim et al. 2001). In the present study, independent of the breed and oocyte yield/antral follicle count, COCs from pre-antral follicles and expanded oocytes tended to be separated from the other groups (Fig. 1) due to substantial differences in their lipid profiles. COCs from expanded oocytes presented high abundance of some ions that were in lower abundance in COCs from pre-antral follicles. Conversely, the ion that was present in low quantity in COCs from expanded oocytes presented high abundance of some ions that were in lower abundance in COCs from pre-antral follicles. Conversely, the ion that was present in low quantity in COCs from expanded oocytes could be related to metabolic status, reduced cytoplasmic membrane content or membrane specialization in oocytes at growing stages compared with oocytes in the antral follicles, because differences in the lipid profile between COCs from pre-antral or

Fig. 3. Tridimensional PLS-DA score plot of representative oocytes from each of the four experimental groups. 1/2 indicus × taurus cows with high AFC (AH group, n = 12, 6 mass spectra) are represented by red triangles; 1/2 indicus × taurus with low AFC (AL group, n = 5, 4 mass spectra) by green triangles; indicus cows with high AFC (NH group; n = 31; 10 mass spectra) by dark blue triangles; and indicus cows with low AFC (NL group; n = 5, 3 mass spectra) by light blue triangles. Each component indicates the percentage of the variability of the data that is explained.

Fig. 4. Line graphic showing the relative ion abundances (y-axis) in the lipid mass spectra of each sample of 1/2 indicus × taurus (AH group samples: A1 to A6; AL group samples: A7 to A10) and indicus (NH group; N1 to N10; NL group: N11 to N13)
expanded follicles were mainly related to the PC lipid species.

Large amounts of intracellular lipids, represented by lipid droplets, are present in the oocyte cytoplasm (Kim et al. 2001). Lipid droplets store neutral lipids, such as TAG, and are surrounded by a monolayer of phospholipids and associated proteins (Bartz et al. 2007). Indeed, the majority of the intracellular lipids in oocytes are TAG (Homa et al. 1986; McEvoy et al. 2000). A metabolic role of TAG in the in vitro maturation of pig and bovine oocytes has been demonstrated (Ferguson and Leese 1999; McEvoy et al. 2000), which indicates that oocytes may use lipids as an energy source, providing ATP for the protein synthesis that is necessary for the continuation of cytoplasmic maturation and meiosis (Kruip et al. 1983). During in vitro maturation, the TAG content of pig oocytes decreases (Sturmey and Leese 2003). Similarly, higher amounts of triglyceride have been measured in immature oocytes compared with in vitro
matured oocytes (Kim et al. 2001; Ferguson and Leese 1999). Conversely, a study has found no lipid variation in bovine oocytes after in vitro maturation (Aardema et al. 2008).

TAG can also be used as a source of energy during early embryo development (Hillman and Flynn 1980; Waterman and Wall 1988; Khandoker and Tsuiji 1998; Spindler et al. 1999; Gomez et al. 2001, 2002). It was reported that in vitro-produced bovine zygotes were able to cleave in the complete absence of exogenous nutrients (Ferguson and Leese 1999). Indeed, substrate oxidation by five- to eight-cell embryos was continuous (Ferguson and Leese 2006), which suggests the use of lipid internal stores. In the present study, the oocytes of indicus females clustered separately from the oocytes of 1/2 indicus × taurus, particularly the NH group, when only the GI, II and III oocytes were considered, whereas the 1/2 indicus × taurus oocytes (AH and AL groups) were mixed (Fig. 3). This difference was due mainly to the greater abundance of four ions in the NH and NL groups compared with the AH and AL groups, most of them corresponded to PC lipid species (Fig. 4). These findings indicate different metabolic rates among indicus and 1/2 indicus × taurus oocytes and that indicus females with high AFC/oocyte yields have an energy store advantage over 1/2 indicus × taurus donors with high and low oocyte yields. These PC lipid species may be associated with lipid droplets and other cellular membranes and have an active role in the metabolism of stored lipids during oocyte maturation.

A recent study (Pontes et al. 2011) demonstrated that oocytes recovered by OPU from indicus donors with high oocyte production produced significantly more embryos and more pregnancies compared with females with low oocyte production. The greater abundance of the four lipid ions observed in the present study could be related to the differences between indicus donors with high and low oocyte yields reported by Pontes et al. (2011). However, great variation in the lipid droplet number in oocytes from the same ovary has been observed (Aardema et al. 2008).

Because PC lipids are involved in the bilayer membrane structure, the present results indicate membrane structural differences between indicus and 1/2 indicus × taurus oocytes from females with high and low AFC/oocyte yields. The physiological significance of the greater abundance of the four lipid species in indicus oocytes compared with 1/2 indicus × taurus oocytes could be related to membrane metabolic rate differences and may contribute to oocyte competence and embryo development. The results also suggest that expanded oocytes may have lipids similar to those of a secretory, Graafian follicle. This intriguing possibility will be further investigated by MALDI-MS with a larger set of samples.

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**Conflict of interest**

Authors declare no conflict of interest.

**Author contributions**


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Author’s address (for correspondence): Marcelo Marcondes Seneda, Laboratório de Reprodução Animal (ReproA), DCV-CCA-Universidade Estadual de Londrina (UEL), Londrina, PR 86051-990 Brazil. E-mail: mseneda@uel.br

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