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Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality

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Abstract

The microenvironment of the developing follicle is critical to the acquisition of oocyte developmental competence, which is influenced by several factors including follicle size and season. The aim of this study was to characterise the metabolomic signatures of porcine follicular fluid (FF) collected from good and poor follicular environments, using high-resolution proton nuclear magnetic resonance (¹H-NMR) spectroscopy. Sow ovaries were collected at slaughter, 4 days after weaning, in summer and winter. The contents of small (3–4 mm) and large (5–8 mm) diameter follicles were aspirated and pooled separately for each ovary pair. Groups classified as summer-small ($n=8$), summer-large ($n=15$), winter-small ($n=9$) and winter-large ($n=15$) were analysed by ¹H-NMR spectroscopy. The concentrations of 11 metabolites differed due to follicle size alone ($P<0.05$), including glucose, lactate, hypoxanthine and five amino acids. The concentrations of all these metabolites, except for glucose, were lower in large FF compared with small FF. Significant interaction effects of follicle size and season were found for the concentrations of glutamate, glycine, *N*-acetyl groups and uridine. Succinate was the only metabolite that differed in concentration due to season alone ($P<0.05$). The FF levels of progesterone, androstenedione and oestradiol were correlated with the concentrations of most of the metabolites examined. The results indicate that there is a distinct shift in follicular glucose metabolism as follicles increase in diameter and suggest that follicular cells may be more vulnerable to oxidative stress during the summer months. Our findings demonstrate the power of ¹H-NMR spectroscopy to expand our understanding of the dynamic and complex microenvironment of the developing follicle.

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Introduction

During the latter stages of folliculogenesis, the cumulus–oocyte complex (COC) is bathed in follicular fluid (FF), which provides an important and specialised microenvironment for *in vivo* oocyte maturation. It is well known that FF consists of exudates from serum and factors produced by the oocyte and somatic compartment. Characterisation of FF can provide useful information about the growth and differentiation of the follicle (Edwards 1974). Various studies performed in mammals clearly demonstrate that FF contains components essential to oocyte maturation and fertilisation and molecules related to follicular cell proliferation and differentiation (Gérard *et al.* 2002, Pinero-Sagredo

et al. 2010). Owing to the close physical relationship between the COC and the FF, analysis of this fluid is of extreme interest for elucidating the determinants of oocyte quality.

Investigation of FF has increased over the last decade but its composition is still relatively unknown. Previous studies have considered the concentration of various hormones (Takahashi *et al.* 2008, Bertoldo *et al.* 2011a), growth factors (Wang *et al.* 2006, Wu *et al.* 2007), reactive oxygen species (Oyawoye *et al.* 2003, Das *et al.* 2006) and other biomarkers (Wallace *et al.* 2012, Yang *et al.* 2012) and have attempted to correlate the biochemistry of FF with oocyte developmental competence and embryo development with varying success. Nonetheless, targeted analysis of certain amino acids and

steroids has revealed interesting correlations with oocyte developmental competence and IVF outcomes (D'Aniello *et al.* 2007, Boxmeer *et al.* 2009, Bertoldo *et al.* 2011a).

The modern domestic sow (*Sus domesticus*) generally breeds throughout the year and is capable of producing two litters annually. However, a reduction in fertility is observed during the late summer and early autumn months in many countries around the world (Love *et al.* 1993, Peltoniemi *et al.* 1999, Bertoldo *et al.* 2009). This phenomenon has been referred to as seasonal infertility and its primary manifestation is increased early pregnancy loss (reviewed by Bertoldo *et al.* (2012)). The mechanisms that underpin this pregnancy loss are highly complex and remain poorly understood. Recent observations have implicated follicle development and ovarian progesterone in the reduction of sow fertility (Bertoldo *et al.* 2011a), and there is clear evidence that oocyte developmental competence is reduced during the seasonal infertility period (Bertoldo *et al.* 2010). Oocytes recovered from large follicles (5–8 mm diameter) in summer had a significantly reduced capacity to form blastocysts compared with those recovered in winter, whereas oocytes recovered from small follicles (3–4 mm diameter) had a limited capacity to form blastocysts regardless of season (Bertoldo *et al.* 2010). Therefore, the seasonal and follicle size differences observed in the sow make it an ideal comparative model to reveal changes in FF composition that support the acquisition of oocyte developmental competence.

Metabolomics is the non-targeted identification and quantification of all the metabolites present in a biological sample, with the aim of measuring the response of an organism to environmental stimuli or modification. In contrast to transcriptomic or proteomic analysis, which observes effects on gene and protein expression, respectively, metabolomics has the advantage of understanding the biological processes being completed as it is capable of providing biological endpoint markers that occur as a result of environmental change or altered gene function (Lindon *et al.* 2004, Baskind *et al.* 2011). By analysing different FF samples, the metabolic profiles obtained can act as fingerprints or signatures for certain physiological states (Lindon *et al.* 2004). High-resolution proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy is a unique tool for studying the composition of biofluids as it provides qualitative and quantitative data of all the metabolites present in a sample (Revelli *et al.* 2009). Using the previously observed seasonal and follicle size effects to provide models of good and poor follicular environments, the aim of this study was to characterise the metabolomic signatures of porcine FF samples collected from small and large follicles in winter and summer.

Materials and methods

Sample collection

FF samples were collected from the ovaries of adult Large White/Landrace cross-bred sows as described by Bertoldo *et al.* (2011a). The sows were sourced from commercial piggeries located in Victoria and South Australia and selected for culling following removal of piglets at weaning (mean parity of 6.0). Only sows culled for reasons unrelated to infertility (referred to as 'non-reproductive' culls), such as lameness, were included in the study. The piglets were weaned 21 days after birth, and the sows were killed at a commercial abattoir 4 days later. This ensured that ovaries were collected during the follicular phase of the oestrous cycle, as a weaning to oestrus interval of 5–7 days is typically exhibited in domestic sows. The ovaries were excised from the reproductive tracts (each ovary pair was kept together in a small sealable plastic bag) and transported to the laboratory in a thermos flask containing saline (0.9% w/v NaCl; Baxter Healthcare, Deerfield, IL, USA) supplemented with 100 U/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml amphotericin (Invitrogen Australia) and maintained at 34–38 °C. Upon arrival, ovaries were washed thoroughly and immersed in saline at room temperature. Ovaries with corpora haemorrhagica or corpora lutea present were excluded from the study. Follicles were measured individually with calipers and aspirated within 2 h of ovary collection using a 21-gauge needle through which constant suction (1.5 l/min) was applied. For each ovary pair, the aspirated material from small follicles (3–4 mm diameter) was pooled in one tube and that from large follicles (5–8 mm diameter) was pooled in another tube. Follicles <3 or >8 mm in diameter or that were observed to have viscous FF (indicating that the LH surge had already occurred) were excluded from the study. Cellular material (COCs and granulosa cells) was removed from the samples by centrifuging them at 3000 g for 20 min and transferring the supernatant (FF) to new tubes. Samples were collected on at least three separate occasions in each season. The volume of FF obtained from each sow ranged from 50 to 1000 μl for small follicles and 400 to 1000 μl for large follicles, but only samples that contained sufficient FF (500 μl) were analysed (summer-small ($n=8$), summer-large ($n=15$), winter-small ($n=9$) and winter-large ($n=15$)). All samples were stored at -20°C within 1 h of follicle aspiration.

Sample preparation

For $^1\text{H-NMR}$ spectroscopy, the FF samples were thawed at room temperature and then centrifuged at 3000 g for 5 min. Samples were prepared by mixing 500 μl FF, 100 μl deuterium oxide (D_2O) solution and 100 μl phosphate buffer to obtain a pH value of 7.4 ± 0.5 . The samples were then transferred to 5 mm NMR tubes (CortecNet, Paris, France) for $^1\text{H-NMR}$ analysis.

$^1\text{H-NMR}$ spectroscopy

The $^1\text{H-NMR}$ spectroscopy was performed on a Bruker DRX-500 spectrometer (Bruker SADIS, Wissembourg, France), operating at 11.7 T, with a broadband inverse probe head

equipped with a Z gradient coil. NMR measurements were performed at 298 K. Conventional ¹H-NMR spectra were recorded with 90° pulse ($p_1=10\ \mu\text{s}$, $pl=0\ \text{dB}$) using a pulse-and-acquire sequence with residual water pre-saturation (single-frequency irradiation during the relaxation delay). ¹H spectra were collected with 128 transients (and eight dummy scans) in 32 K data points with a spectral width of 7500 Hz and a recycling time of 15 s. Carr-Purcell-Meiboom-Gill (CPMG) spin echo spectra were carried out with 80 ms total echo times and 32 K data points. This spin echo sequence avoided broad short T_2 resonance. Sample shimming was performed automatically on the water signal. Spectra were processed using WinNMR version 3.5 Software (Bruker Daltonik, Karlsruhe, Germany). Prior to Fourier transformation, the free induction decay (FID) signals were zero-filled to 64 K data points, which provided sufficient data points for each resonance and a line-broadening factor of 0.3 Hz was applied. All spectra were corrected for phase distortion and the baseline was manually corrected for each spectrum. To quantify this, the electronic reference to *in vivo* concentrations (ERETIC) signal (Barantin *et al.* 1997) was used. It was generated using one of the frequency channels of the spectrometer and was sent to the carbon channel of the probe during the acquisition of the NMR signal (Akoka *et al.* 1999). The ERETIC signal consists of an exponentially decaying function that mimics the behaviour of a FID signal. The NMR parameters of this ERETIC FID were under control. The ERETIC peak is provided as a quantitative reference signal that has the same area in all spectra (Barantin *et al.* 1997). Spectral ¹H assignments were made based on the literature values of chemical shifts in various media and biofluids (Nicholson *et al.* 1995, Mailliet *et al.* 1998). The ¹H-NMR spectra were referenced to the lactate resonance at 1.33 p.p.m. and automatically reduced to ASCII files using AMIX Software package (Analysis of MIXture, version 3.1.5, Bruker Biospin, Karlsruhe, Germany). The regions from 4.20 to 6.4 p.p.m. were removed from each spectrum to eliminate baseline effects of imperfect water saturation. Spectral intensities were scaled to the total intensity and reduced to equidistant integrated regions of 0.001 p.p.m. (buckets) over the chemical shift range of 0.7–9.5 p.p.m. Before the multivariate analysis, the NMR spectral data set was pre-processed using peak alignment algorithm icoshift (<http://www.models.life.ku.dk>; Savorani *et al.* 2010) to minimize spectral peak shift due to residual pH differences within samples. The corresponding realigned bucket tables were then exported to the SIMCA-P⁺ Software (version 12.0, Umetrics, Umea, Sweden) for analysis.

Data analyses

A supervised method, partial least-squares discriminant analysis (PLS-DA), was performed using SIMCA-P⁺ Software (Eriksson *et al.* 2008). Data were scaled using unit Pareto (Par) scaling prior to PLS-DA. PLS-DA is a multivariate projection method that can be used to classify samples according to their common spectral characteristics. PLS-DA takes advantage of class information to attempt to maximize the separation between groups of observations. A plot of the first two principal components (scores plot) provided the most effective 2D

representation of the information contained in the data set. The overall quality of the models was judged by cumulative R^2 and the predictive ability by cumulative Q^2 extracted according to the internal cross-validation default method of SIMCA-P⁺ Software.

An orthogonal PLS-DA (OPLS-DA) as a supervised model of classes, was also performed using SIMCA-P⁺ Software (Cloarec *et al.* 2005, Eriksson *et al.* 2008, Sadeghi-Bazargani *et al.* 2011). Data were scaled using unit Par scaling prior to OPLS-DA. OPLS-DA is a prediction and regression method that finds information in the X data (variables) that is related to known information, the Y data (classes). OPLS-DA takes advantage of class information to attempt to maximize the separation between groups of observations. The overall quality of the models was judged by cumulative R^2 and the predictive ability by cumulative Q^2 . R^2 is defined as the proportion of variance in the data explained by the model and indicates goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability. In addition, to identify putative biomarkers from the data, the contribution plot was used. The contribution plot provides information on the variables that influence any observed clustering of samples (negative values refer to variables associated with one group and positive values refer to variables associated with the second group).

Statistical analyses

A linear mixed model was fitted to the data using the statistical software package GenStat 14th edition (VSN International, Ltd., Hemel Hempstead, UK). For each metabolite, the effects of 'follicle size' and 'season' were assessed for significance ($P<0.05$), as was the interaction effect. The term 'pig', which specified individual animals, was included as a random term. Residual plots were examined to confirm model assumptions. When an interaction effect was detected, approximate least significant differences (LSDs) were used to compare pairs of means.

Correlations of metabolite concentrations with steroid concentrations were initially assessed by including progesterone, androstenedione and oestradiol (E_2) in a separate linear mixed model series. As the steroid concentrations were strongly correlated with each other, the two steroids that are found to be least significantly correlated with the metabolite were then sequentially removed from the model. The correlation coefficient (r) was then calculated for the metabolite and each steroid.

Results

FF ¹H-NMR spectroscopic profiles

Representative ¹H-NMR spectra of FF samples are shown in Fig. 1. The FF samples contained low-molecular-weight metabolites including amino acids (alanine, glycine, phenylalanine and tyrosine), creatine-creatinine, organic acids (lactate, acetate, pyruvate and formate), carbohydrates (glucose), trimethylamine groups, *N*-acetyl groups and some lipid groups.

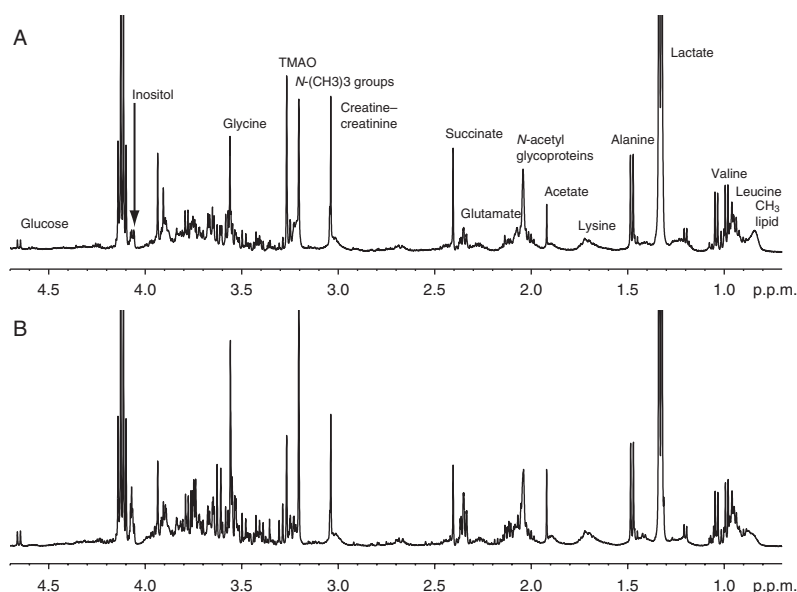


Figure 1 Representative $^1\text{H-NMR}$ spectra (500 MHz) of porcine follicular fluid samples collected from large follicles in winter (A) and small follicles in summer (B).

Multivariate analysis of the $^1\text{H-NMR}$ spectral data

The scores scatter plot resulting from the PLS-DA of the $^1\text{H-NMR}$ spectral data is shown in Fig. 2A ($R^2Y=0.47$ and $Q^2=0.29$). The plot shows a slight follicle size effect (open vs closed symbols) and a slight season effect (squares vs circles) on the metabolic profile of FF. However, the goodness of prediction of the model ($Q^2=0.29$) was quite low. Figure 2B shows that the OPLS-DA of the $^1\text{H-NMR}$ spectral data achieved a clearer separation between the small FF and large FF groups ($R^2Y=0.685$ and $Q^2=0.50$). Figure 2C shows that the OPLS-DA of the $^1\text{H-NMR}$ spectral data also achieved a more obvious separation between the summer FF and winter FF groups ($R^2Y=0.69$ and $Q^2=0.52$). The corresponding contribution plots (not shown) were used to identify the metabolites responsible for the discriminations. The areas of the spectral peaks assigned to these metabolites were then used to quantitate their concentrations in each FF sample.

Effects of follicle size and season on the concentrations of metabolites in FF

Follicle size was found to affect the concentrations of 11 metabolites independent of season (Fig. 3). Interestingly, the concentrations of all but one of these metabolites, glucose, were significantly greater in FF from small follicles (3–4 mm diameter; small FF) than those in FF from large follicles (5–8 mm diameter; large FF). These ten metabolites included acetate, lactate, hypoxanthine, inositol, trimethylamine and five amino acids (alanine, leucine, lysine, methionine and phenylalanine). Glucose was the only metabolite affected by follicle size alone that had an increased concentration in large FF compared with

small FF ($P<0.05$). The corresponding percentage differences in metabolite concentrations between large FF and small FF, for those metabolites significantly affected by follicle size alone, are shown in Fig. 4. The concentration of hypoxanthine in large FF was 44% lower than that in small FF, whereas the concentration of glucose in large FF was 16% greater than that in small FF.

The only metabolite whose concentration was significantly affected by season, independent of follicle size, was succinate. The concentration of succinate in FF collected in winter (winter FF, 0.253 ± 0.015 mM) was significantly greater than that in FF collected in summer (summer FF, 0.218 ± 0.007 mM), representing a 17% difference. The winter FF and summer FF concentrations of three other metabolites, glucose (0.890 ± 0.048 and 0.785 ± 0.044 mM respectively; $P=0.095$), methionine (0.136 ± 0.008 and 0.114 ± 0.008 mM respectively; $P=0.077$) and lipid methyl (0.876 ± 0.041 and 0.955 ± 0.026 mM respectively; $P=0.093$), tended to differ.

Significant interaction effects between follicle size and season were found for the concentrations of four metabolites (Fig. 5). Glutamate concentrations were greater in small FF compared with large FF, and the difference was greater in summer ($P<0.05$). The concentrations of glycine and uridine in small FF collected in summer were greater than those in small FF collected in winter and were also greater than those in large FF collected in either season ($P<0.05$). Likewise, the concentration of *N*-acetyl groups in small FF collected in summer was greater than that in small FF collected in winter ($P<0.05$), but the concentrations in large FF collected in either season were intermediate.

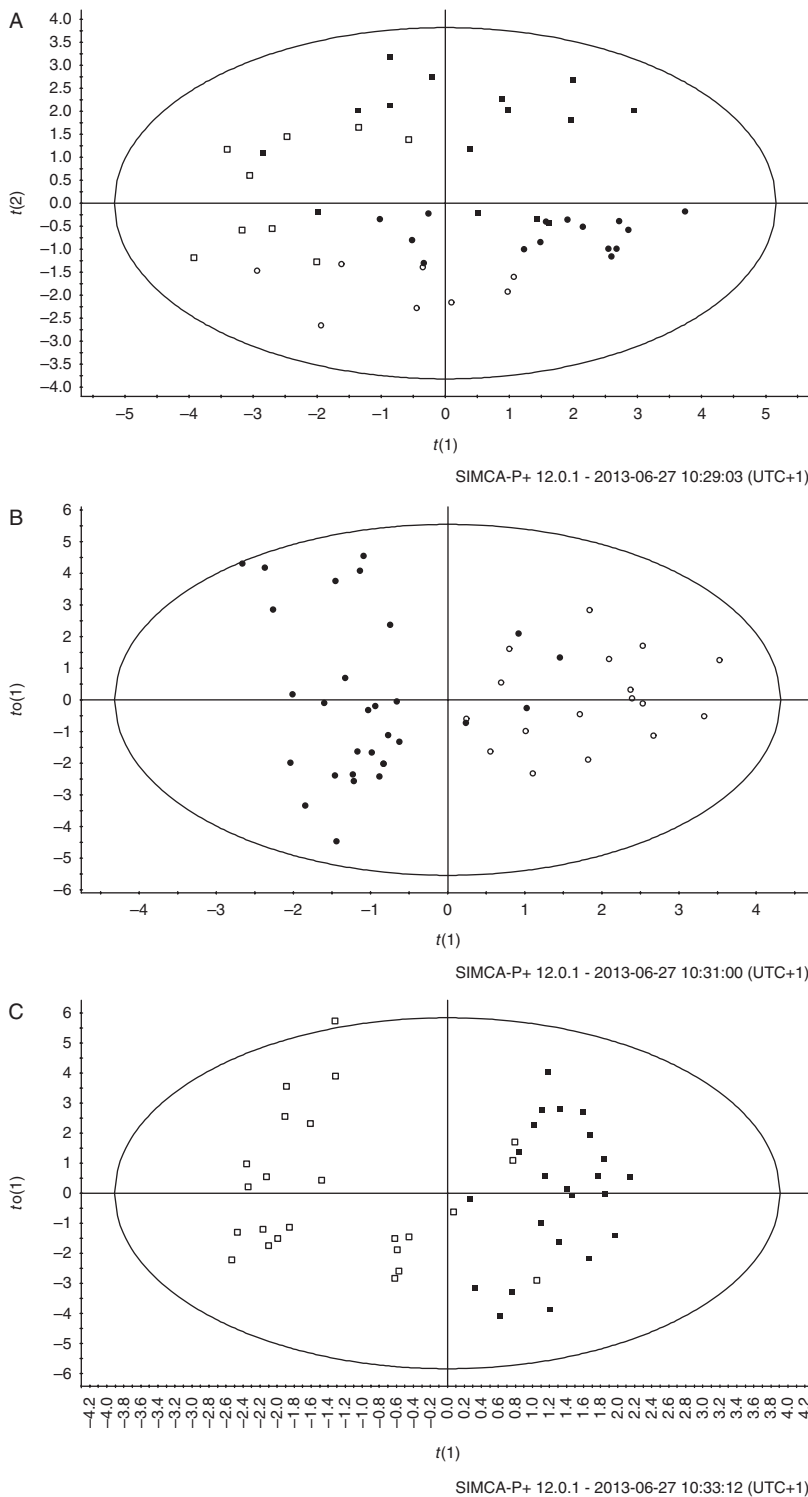


Figure 2 (A) PLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine follicular fluid (FF) samples of the winter-small ($n=9$; open squares), winter-large ($n=15$; closed squares), summer-small ($n=8$; open circles) and summer-large ($n=15$; closed circles) groups. The R^2Y and Q^2 values are 0.47 and 0.29 respectively, showing a slight follicle size effect on axis 1 (open vs closed symbols) and a slight season effect on axis 2 (squares vs circles) on the metabolic profiles. (B) OPLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine FF samples of the small ($n=17$; open circles) and large ($n=30$; closed circles) follicle size groups. The two populations are well separated with R^2Y and Q^2 values of 0.685 and 0.50 respectively, showing a clear effect of follicle size on the metabolic profiles. (C) OPLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine FF samples of the summer ($n=23$; closed squares) and winter ($n=24$; open circles) groups. The two populations are well separated with R^2Y and Q^2 values of 0.69 and 0.52 respectively, showing a clear effect of season on the metabolic profiles.

Relationships between the concentrations of metabolites and steroids in FF

A previous analysis of the samples assessed in this study showed a significant interaction effect of follicle size and season on the FF concentration of progesterone and that FF concentrations of androstenedione and E₂ were

affected by follicle size but not by season (Bertoldo *et al.* 2011a). Further analysis of the metabolite concentrations revealed numerous correlations with FF steroid concentrations (Table 1). The concentrations of ten metabolites, acetate, alanine, glutamate, hypoxanthine, lactate, leucine, lysine, methionine,

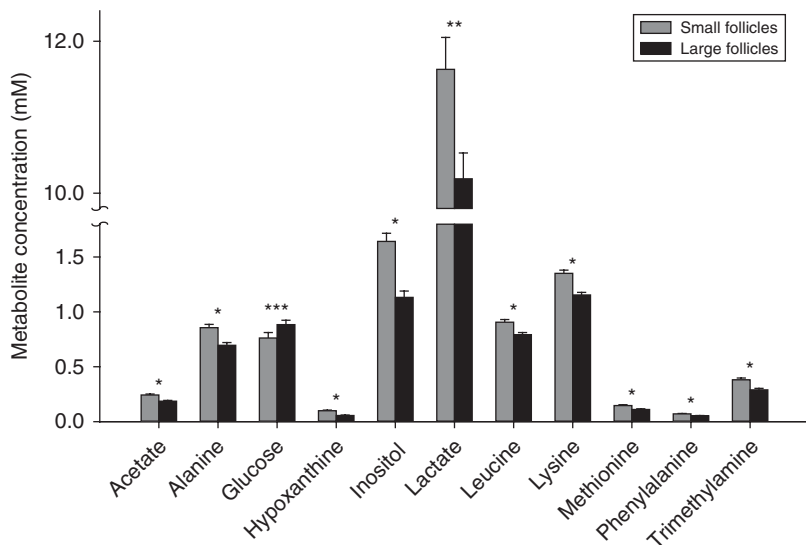


Figure 3 Porcine follicular fluid metabolites that differed significantly in concentration between small (grey bars) and large (black bars) follicles. Asterisks indicate the *P* value determined for the differing concentration values (means ± S.E.M.) for each metabolite (**P*<0.001, ***P*<0.01 and ****P*<0.05).

phenylalanine and succinate, were most strongly correlated with the concentration of E₂, and all were negatively correlated. The concentrations of four metabolites, glycine, inositol, trimethylamine and uridine, were most strongly correlated with the concentration of progesterone, and all were negatively correlated. The concentration of only one metabolite, trimethylamine oxide, was correlated with the concentration of androstenedione. In contrast with the other correlations, the concentrations of trimethylamine and androstenedione were positively correlated.

Discussion

Using ¹H-NMR spectroscopy, this study demonstrated that the metabolomic signatures of porcine FF from small and large antral follicles differ markedly and are

influenced by the season. As the developmental potential of oocytes recovered from large follicles has been shown to be greater than that of oocytes recovered from small follicles in numerous species (Crozet *et al.* 1995, Marchal *et al.* 2002, Iwata *et al.* 2004, Kauffold *et al.* 2005, Lequarre *et al.* 2005, Bagg *et al.* 2007, Grupen *et al.* 2007), the differences found may provide insight into the cellular processes involved during the acquisition of oocyte developmental competence. Similarly, the observed differences may provide clues to the possible cause of reduced oocyte quality in sows during the period of seasonal infertility (Bertoldo *et al.* 2010, 2011b). Importantly, the seasonally induced differences in the FF metabolomic signatures differed considerably from those attributed to follicle size, suggesting that disparate mechanisms are responsible for assuring that good oocyte quality is acquired.

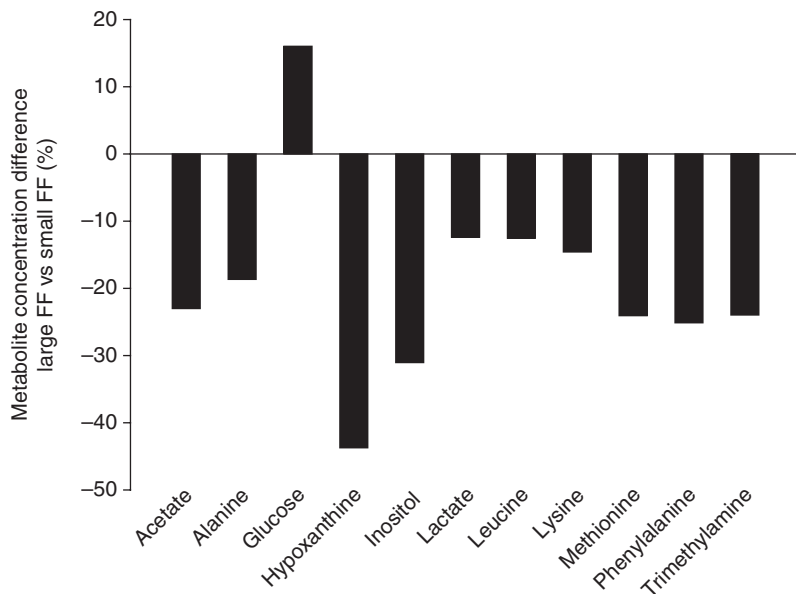


Figure 4 Porcine follicular fluid (FF) metabolites that were found to differ significantly in concentration between small and large follicles as shown in Fig. 3. The value shown for each metabolite is the percentage that the mean concentration in FF from large follicles (5–8 mm) differed from the mean concentration in FF from small follicles (3–4 mm).

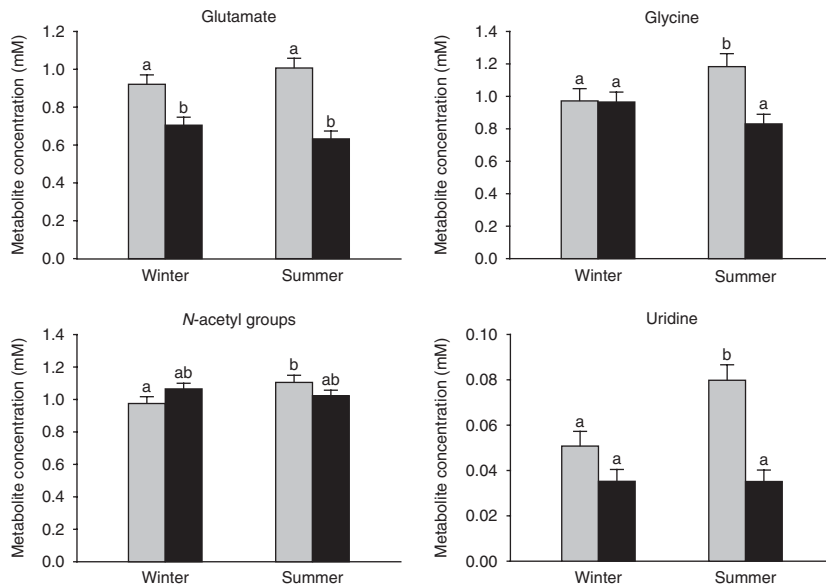


Figure 5 Concentrations (mean ± S.E.M.) of metabolites in follicular fluid samples collected from small (grey bars) and large (black bars) follicles in winter and summer, where an interaction effect of season and follicle size was detected using a linear mixed model. Approximate least significant differences were used to compare pairs of means. Bars with different letters differ significantly ($P < 0.05$).

Our analysis revealed an effect of follicle size, independent of season, on the concentrations of 11 metabolites. Only glucose levels were greater in large follicles compared with small follicles, with the levels of acetate, lactate, hypoxanthine, inositol, trimethylamine and the amino acids alanine, leucine, lysine, methionine and phenylalanine being lower. The finding that glucose concentration increased as follicular diameter increased is consistent with analyses of sheep (Ying *et al.* 2011), cattle (Leroy *et al.* 2004) and buffalo (Nandi *et al.* 2008) FF, but contrary to findings in camels (Ali *et al.* 2008) and horses (Collins *et al.* 1997, Gérard *et al.* 2002). Furthermore, Leroy *et al.* (2004) also observed a concomitant reduction in lactate levels as follicle size increased. The opposing change in the levels of glucose and lactate may be due to a reduction in glucose

catabolism and lactate secretion by granulosa cells as follicular development progresses. However, given the importance of glucose during oocyte maturation, this seems unlikely. Glucose is metabolised by COCs via the glycolytic pathway to provide pyruvate for energy generation (Sutton-McDowall *et al.* 2010). Glucose is also utilized by the pentose phosphate pathway to regulate nuclear maturation and redox state and by the hexosamine biosynthesis pathway to provide substrates required for processes such as cumulus expansion and cell signalling (Sutton-McDowall *et al.* 2010). An alternative explanation is that as follicle size increases, there are relatively fewer granulosa cells consuming glucose and secreting lactate into the relatively greater volume of FF. While it can be argued that post-mortem turnover of glucose to lactate by anaerobic glycolysis

Table 1 Correlations between metabolite and steroid concentrations in porcine follicular fluid.

Metabolite	Effect ^a	Progesterone ^b	Androstenedione ^b	Oestradiol ^b
Acetate	F	-0.3988 (0.009)	-0.3922 (0.010)	-0.4779 (0.001)
Alanine	F	-0.3148 (0.042)	-0.4735 (0.002)	-0.5456 (<0.001)
Glucose	F	0.1450 (0.360)	0.1551 (0.327)	0.0985 (0.535)
Hypoxanthine	F	-0.5210 (<0.001)	-0.5123 (<0.001)	-0.5624 (<0.001)
Inositol	F	-0.5243 (<0.001)	-0.2997 (0.054)	-0.3181 (0.040)
Lactate	F	-0.1979 (0.209)	-0.3993 (0.009)	-0.4739 (0.002)
Leucine	F	-0.2379 (0.129)	-0.4545 (0.003)	-0.5080 (<0.001)
Lysine	F	-0.3930 (0.010)	-0.5006 (<0.001)	-0.5304 (<0.001)
Methionine	F	-0.2383 (0.129)	-0.3347 (0.030)	-0.4498 (0.003)
Phenylalanine	F	-0.2496 (0.111)	-0.5668 (<0.001)	-0.5956 (<0.001)
Trimethylamine	F	-0.4136 (0.007)	-0.1187 (0.454)	-0.1102 (0.487)
Glutamate	F×S	-0.4040 (0.008)	-0.5086 (<0.001)	-0.5745 (<0.001)
Glycine	F×S	-0.3590 (0.020)	-0.1894 (0.230)	-0.3132 (0.043)
N-acetyl groups	F×S	-0.0240 (0.880)	-0.0063 (0.968)	0.0224 (0.888)
Uridine	F×S	-0.4480 (0.003)	-0.3639 (0.018)	-0.3656 (0.017)
Succinate	S	-0.1842 (0.243)	-0.2074 (0.188)	-0.3127 (0.044)
Trimethylamine oxide	-	-0.0635 (0.690)	0.3449 (0.025)	0.1554 (0.326)

^aThe observed effect on metabolite concentration was due to follicle size alone (F), season alone (S) or an interaction effect (F×S). ^bValues shown are the correlation coefficients (*r*) describing the metabolite–steroid relationships, with the corresponding *P* values in parentheses. For each metabolite, the value for the strongest significant metabolite–steroid correlation is shown in bold.

(Leroy *et al.* 2004) may have contributed to the observed changes in glucose and lactate concentrations, any follicular cell death that may have occurred prior to sampling does not explain the seasonal and interaction effects. Furthermore, as already mentioned, the changes in glucose and lactate levels were consistent with those reported by Leroy *et al.* (2004) in cattle FF, which was sampled following transportation of ovaries on ice (4 °C).

When the interaction effects of follicle size and season were also taken into consideration, the increase in glucose concentration in large FF corresponded to a decrease in the concentrations of numerous amino acids, including alanine, glutamate, glycine, leucine, lysine, methionine and phenylalanine. Hong & Lee (2007) reported a very similar effect of follicle size on the amino acid concentrations in porcine FF, even though the follicle size categories differed slightly to those described here. The glucogenic amino acids can be degraded to pyruvate or an intermediate of the tricarboxylic acid (TCA) cycle for the production of glucose under conditions of low glucose, and the ketogenic amino acids can be converted to acetoacetate and acetyl-CoA when energy sources are low. Acetate, another metabolite that decreased in concentration as follicle size increased, can also be converted to acetyl-CoA, which may in turn be utilized by the TCA cycle. A previous study in mares showed that the FF concentrations of alanine and acetate also decreased during follicular growth and preovulatory follicular maturation respectively (Gérard *et al.* 2002). Taken together, the results indicate that the TCA cycle, a key energy-generating metabolic pathway, is employed by granulosa cells to a greater extent in large follicles than in small follicles and represents an increase in the energy demands of the growing follicle.

The importance of the TCA cycle during the acquisition of oocyte quality is further implicated by the fact that succinate, the only metabolite found to differ in concentration between seasons independently of follicle size, was at lower levels in summer FF compared with winter FF. Succinate is an intermediate of the TCA cycle and donates electrons to the electron transport chain when oxidized to fumarate. Lower levels of succinate in summer FF may therefore result in follicular cells having a reduced capacity to generate energy via oxidative phosphorylation.

Large FF also had lower concentrations of trimethylamine, inositol and hypoxanthine compared with small FF. Gérard *et al.* (2002) reported a similar change in the FF level of trimethylamine between the late dominant and preovulatory stages in mares and proposed that the change in trimethylamine concentration was linked to polyamine metabolism (Gérard *et al.* 2002). Inositol is a precursor of the inositol phospholipids, which regulate many cellular processes in response to hormonal or other types of stimuli. The results of a study in women undergoing IVF treatment indicated that the FF level of inositol was positively associated with oocyte

developmental potential (Chiu *et al.* 2002). Therefore, the effect of follicle size on inositol concentration in this study seems counterintuitive, although clearly the conditions of FF sampling were very different to those of the IVF study, in which FF was collected well after the LH surge. In this study, the FF samples were collected from individual sows slaughtered for non-reproductive reasons (e.g. lameness) 4 days after weaning, when the vast majority of sows (about 90%) (Langendijk *et al.* 2000) experience the onset of oestrus. Therefore, the FF metabolomic profiles reported here are representative of the microenvironments that oocytes are exposed to during the follicular phase of the cycle, just prior to the LH surge.

The concentration of hypoxanthine in large FF was 44% lower than that in small FF, the largest relative difference in concentration of all the metabolite comparisons. A previous NMR analysis of porcine FF failed to detect the presence of hypoxanthine (Gosden *et al.* 1990), but the concentrations reported here are still less than a tenth of that determined previously using u.v. absorption spectra and ion-exchange and HPLC (Downs *et al.* 1985). Hypoxanthine is a non-specific phosphodiesterase inhibitor that prevents oocyte meiotic resumption by maintaining elevated intra-oocyte levels of cAMP (Downs *et al.* 1985). A lower concentration of hypoxanthine in large FF therefore seems logical, as this would allow the resumption of oocyte meiosis to occur more readily in response to the impending LH surge.

In small FF, the concentrations of uridine and *N*-acetyl groups were greater in summer than in winter. The increased concentration of uridine in small FF during summer indicates that there was an increased level of circulating uridine or a decreased uptake of uridine by granulosa cells. Uridine is the primary circulating pyrimidine in humans (Wurtman *et al.* 2000). Following cellular uptake, uridine is converted to UTP, which can then be utilized for the synthesis of RNA, phosphocholine (Cansev *et al.* 2008) or glycogen via glycogenesis. The concomitant increase in the concentration of *N*-acetyl groups, which are associated with glycoproteins, glycolipids and important antioxidant constituents, may be indicative of a follicular environment that was more sensitive to oxidative stress and/or increased inflammation and cell damage (Kolwijck *et al.* 2009, Sun *et al.* 2012).

All the FF samples analysed in this study were the remaining FF after separate, smaller volumes of each sample had been removed to assay for the concentrations of progesterone, androstenedione and E₂ in a previous study (Bertoldo *et al.* 2011a). As follicle size and season were found to exert effects on the concentrations of steroids in these FF samples (Bertoldo *et al.* 2011a), the differences in metabolite concentrations would be expected to correlate with the steroid changes previously observed. In the majority of cases (10 of 15 metabolites), the metabolite concentrations in

FF were most strongly correlated with the concentration of E₂. The associations were negative, indicating that as E₂ production increased in the developing follicle, concentrations of these metabolites fell. The concentrations of four metabolites, including two of the metabolites found to be greater in small FF during the summer months, were most strongly correlated with the concentration of progesterone. Again, the associations were negative. Reduced production of progesterone by follicular cells both *in vivo* (Grupen *et al.* 2003, Bagg *et al.* 2007, Bertoldo *et al.* 2010) and *in vitro* (Grupen & Armstrong 2010) has been associated with reduced oocyte quality in pigs. Whether changes in steroid production altered the metabolism of follicular cells, or whether the metabolic environment influenced follicular steroidogenic activity, requires further investigation.

The complexity of the follicular environment has made the analysis of FF extremely challenging. Numerous methods have been employed to characterise components of FF in efforts to increase our understanding of oocyte maturation and identify predictors of oocyte quality. To date, there are relatively few reports of the use of NMR spectroscopy to analyse FF samples (reviewed by Baskind *et al.* (2011)), and to our knowledge, there is only one other report of this method being used to determine the composition of porcine FF (Gosden *et al.* 1990). Recent reports of the use of this technology to identify potential biomarkers of oocyte quality (Pinero-Sagredo *et al.* 2010, McRae *et al.* 2012, Wallace *et al.* 2012) and polycystic ovarian syndrome (Atiomo & Daykin 2012) in FF collected from women undergoing fertility treatments highlight its potential. Our findings demonstrate the effectiveness of ¹H-NMR spectroscopy in detecting metabolite differences in intact, untreated FF collected from individual animals and extend the knowledge gained from ¹H-NMR studies in other species. It should be noted that the small FF and large FF samples collected from each animal were not derived from individual follicles but consisted of fluid aspirated from all the small (3–4 mm diameter) and large (5–8 mm diameter) antral follicles respectively in the ovary pair. Follicular dynamics in the pig obviously differ from that in monovular species, in which only the dominant follicle ovulates. Nevertheless, given the variation in oocyte quality, one would assume that minor differences existed between individual follicles of the same size category. Despite this, a large number of metabolite differences were found between groups.

In conclusion, the current study used a model that represented good and poor follicular environments to reveal differences in the metabolomic signatures of FF that may be associated with the acquisition of oocyte developmental competence. Follicle size was found to affect the concentrations of numerous metabolites that can be linked to the TCA cycle, indicating that there is a distinct energy-generating shift during the final stages

of follicle development. The resumption of meiosis in oocytes of large follicles would also be facilitated by the observed decrease in the concentration of hypoxanthine. The reduction in oocyte quality during the period of seasonal infertility appears to involve metabolite changes in FF that are very different from those associated with follicle size. We propose that the effect of season on oocyte quality is in part due to a follicular environment that is less able to support cells exposed to oxidative stress. Finally, for nearly all the metabolites shown to differ in concentration in this study, strong correlations with steroid levels were found. While further studies are needed to clarify the effects of the observed metabolite changes on oocyte quality, these findings clearly demonstrate the power of ¹H-NMR spectroscopy to expand our understanding of the dynamic and complex microenvironment of the developing follicle.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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